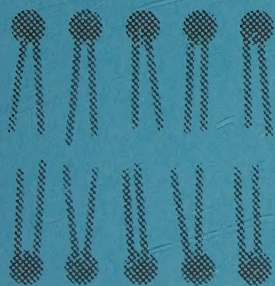


# PARTITION OF MEMBRANES IN AQUEOUS TWO-PHASE SYSTEMS

a study with liposomes as  
a model membrane

by  
**Eva Eriksson**



**Lund 1981**





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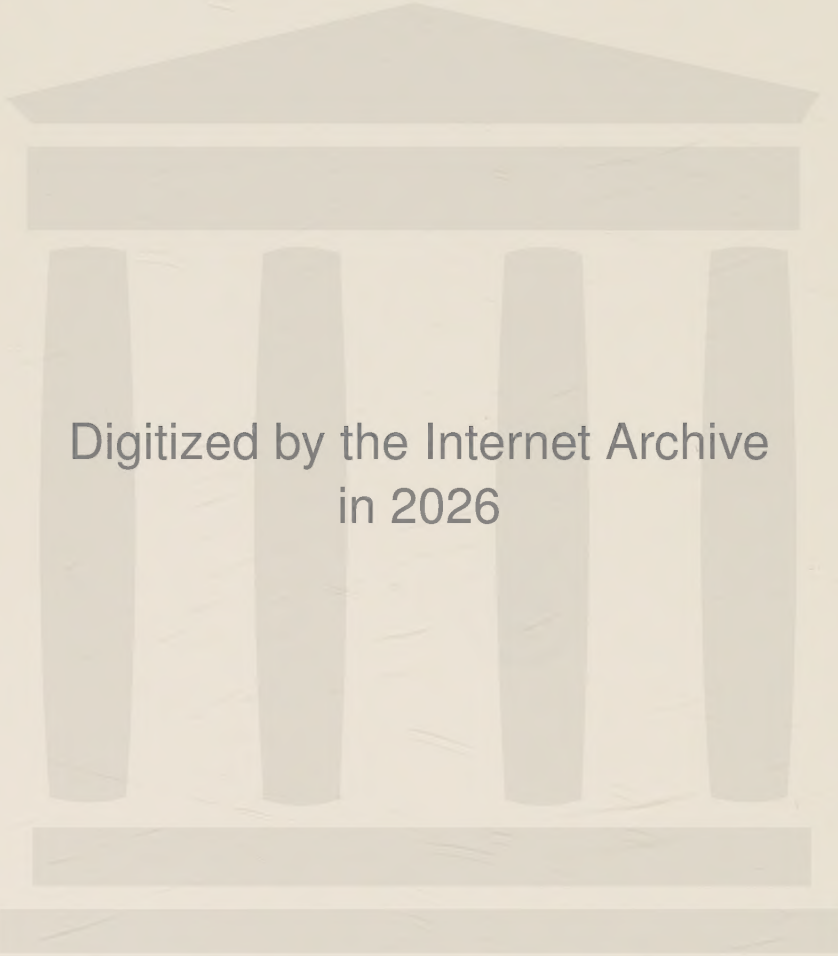
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**Lund 1981**



To my dear parents and  
to Mats and Daniel.



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This thesis is based on the following papers which will be referred to in the text by their Roman numerals:

- I     Hydrophobic Surface Properties of Erythrocytes Studied by Affinity Partition in Aqueous Two-Phase Systems.

Eva Eriksson, Per-Åke Albertsson and Göte Johansson  
Mol.Cell.Biochem.10(1976)123-128.

- II    The Effect of the Lipid Composition on the Partition of Liposomes in Aqueous Two-Phase Systems.

Eva Eriksson and Per-Åke Albertsson.  
Biochim.Biophys.Acta 507(1978)425-432.

- III   Hydrophobic Affinity Partition of Liposomes in Aqueous Two-Phase Systems.

Eva Eriksson.

Accepted for publication in J.Chromatogr.1980.



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## INTRODUCTION.

Aqueous two-phase systems consisting of the two polymers dextran and poly(ethylene glycol) have been widely used in the separation and study of cells and membrane particles /1-5/. Both the upper, poly(ethylene glycol)-rich phase and the lower, dextran-rich phase have a high content of water, 80-95%. The possibility of including salts in desired concentrations make the phase systems very suitable for biological materials. For a better separation the liquid-liquid extraction may be performed in a multistep procedure called counter-current distribution.

Some examples of partitioned cells or cellular particles are erythrocytes /6/, mitochondria/7,8/, chloroplasts/9-11/, plasma membranes/12/, HeLa cells/13/, yeast cells/14/, ribosomes/15/, algae/16/, bacteria/17,18/, synaptic vesicles/19,20/ and leukocytes /21/.

Partition in two-phase systems is based on differences in surface properties, in contrast to several other separation methods which utilize differences in size or density. The specificity for membranes with this method may be improved by using polymer-bound hydrophobic ligands which selectively bind to membrane surfaces. The aim of this work has been to investigate surface properties that determine the partition of mem-

brane particles.

The partition behaviour of erythrocytes from different species has been examined /I/. Two groups of erythrocytes could be distinguished. They differed in their membrane content of sphingomyelin and phosphatidylcholine. A correlation between the partition of erythrocytes and the ratio of poly-/mono-unsaturated fatty acids in their membranes has also been reported /22/.

In order to further study factors that will determine the partition of membranes in aqueous two-phase systems, liposomes were used as model membranes /II, III/. The effect of variations in the polar head group region of the liposomes was studied by partition of liposomes consisting of phosphatidylcholine, phosphatidylserine, phosphatidylinositol or sphingomyelin. The influence of the hydrophobic part of the bilayer on the partition was investigated using phosphatidylcholine liposomes in which the degree of unsaturation of the fatty acids was varied. It was concluded from these investigations that the polar head group of the phospholipid plays a dominant role in determining the partition of liposomes while the degree of unsaturation of the acyl groups is of less importance.

Hydrophobic affinity partition with fatty acids covalently attached to poly(ethylene glycol) can selectively attract membrane particles to the upper poly(ethylene glycol)-rich phase /23, 24/. The fatty acids suppo-

sedly dip down into the membranes and in that way interact with the hydrophobic interior of the membrane. To study the mechanism behind this interaction, liposomes with different degrees of unsaturation and with varying lengths of the phospholipid fatty acids were partitioned in phase systems containing poly(ethylene glycol)-bound fatty acids of different lengths/III/. Also in this case the polar head group of the phospholipids was the dominant factor in determining the partition of liposomes, while the hydrophobic part was of less importance.

The effect of incorporated molecules, such as cholesterol and pigments, on the partition of liposomes in aqueous two-phase systems has also been investigated.



## A REVIEW OF THE LITERATURE.

### 1 The structure of membranes.

Membranes are composed largely of protein and lipid /25/. The ratio (w/w) of protein to lipid varies from 0.25 in myelin to 3.0 in bacterial membranes/26/. A 1:1 ratio (w/w) may be regarded as typical. In addition, small amounts of carbohydrates (less than 5%) are present and traces of RNA (less than 0.1% ) may also be found. The lipid content of membranes is responsible for such properties as high electrical resistance and impermeability to ions and other polar substances. Because of the lipid content non-polar materials often pass through membranes readily. Under the electron microscope the typical membrane appears to be approximately 7 nm thick, but low angle X-ray diffraction suggests a thickness closer to 11 nm.

In 1925 Gorter and Grendel calculated that the erythrocyte membrane contained just enough lipid to form a layer 3-4 nm thick around the cell/27/. The well-known lipid-bilayer model for membrane structure was proposed by Danielli and Dawson in 1935/28/. In this model hydrophilic proteins coated each side of the lipid bilayer. This model was later advanced as the unit membrane model by Robertson in 1959/29/. The fluid mosaic model for membrane structure with alternating globular

proteins and phospholipid bilayer was proposed by Singer and Nicolson in 1972 /30/.

A large number of lipids have been identified in membranes. The distribution of different kinds of lipids varies markedly between membranes from different sources. It is therefore difficult to generalize about membrane composition. However, phospholipids are apparently always present and they make up from about 10% to over 90% of the total lipid (table I).

Table I.

Estimated chemical composition of some membranes.<sup>1</sup>

| Compound                  | % of total dry weight of membrane |         |                               |                   |        |                  |
|---------------------------|-----------------------------------|---------|-------------------------------|-------------------|--------|------------------|
|                           | Myelin<br>rod                     | Retinal | Eryth-<br>rocyte <sup>2</sup> | Mitoch-<br>ondria | E.Coli | Chloro-<br>plast |
| Protein                   | 22                                | 59      | 60                            | 76                | 75     | 48               |
| Total lipid               | 78                                | 41      | 40                            | 24                | 25     | 52               |
| PC                        | 8                                 | 13      | 7                             | 9                 |        |                  |
| PE                        | 12                                | 7       | 7                             | 8                 | 18     |                  |
| PS                        | 7                                 | 3       | 3                             |                   |        |                  |
| PI                        | 1                                 |         |                               | 1                 |        |                  |
| PG <sup>3</sup>           |                                   |         |                               |                   | 4      |                  |
| CL                        |                                   |         |                               | 4                 | 3      |                  |
| SM                        | 6                                 | 1       | 7                             |                   |        |                  |
| GL <sup>4</sup>           | 22                                | 10      |                               |                   |        | 23               |
| CH <sup>5</sup>           | 17                                | 2       | 9                             |                   |        |                  |
| Total PL                  | 33                                | 27      | 24                            | 23                | 25     | 5                |
| PL as % of<br>total lipid | 42                                | 66      | 60                            | 94                | >90    | 9                |

1) The table is taken from /26/.

2) human erythrocytes

3) phosphatidylglycerol

4) glycolipid

5) cholesterol

For other abbreviations see next page.

Five types of phospholipids (PL) dominate: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), cardiolipin (CL) and sphingomyelin (SM). Small amounts of phosphatidylinositol (PI) also occur.

There are large variations between the outside and the inside of many membranes. In erythrocytes phosphatidylcholine and sphingomyelin are mostly on the outer surface while the other phospholipids are on the inner surface of the membrane/31,32/. Phosphatidylcholine predominates in erythrocytes from several species but is replaced by sphingomyelin in others/31/. Glycolipids are presumably exposed on the outer surface of the membrane with the attached carbohydrate groups projecting into the surrounding medium.

The erythrocyte membrane is one of the most extensively studied membranes due to the ease of isolation, purity and quantity of material available. It has also been possible to isolate sealed inside-out erythrocyte vesicles by partition in two-phase systems/33/. The erythrocyte membrane is composed of approximately 20 proteins and glycoproteins (for reviews see /25,31/). The majority of these are considered to be integral components of the membrane, i.e. they are solubilized by detergents, chaotropic agents, bile salts, organic solvents or harsh pH treatments. Approximately 20-25% of the membrane proteins are peripheral (eluted by low ionic strength media). The peripheral proteins are mainly spectrin (also called tecktin A) and actin. Both

are probably located at the inner membrane surface. Of the other protein components in the erythrocyte membrane, two integral proteins are of particular interest. These are component *a* and the major sialoglycoprotein (also called MN-glycoprotein or glycophorin). These proteins are the major proteins exposed to the external environment and are receptors for a variety of viruses, lectins and specific antibodies. The major glycoprotein contains about 60% (w/w) carbohydrate and carries a high negative charge because of a high content of sialic acid.



## 2. Liposomes.

Liposomes are microscopic structures consisting of one or more concentric lipid bilayers in an onion-like arrangement enclosing an equal number of aqueous compartments. These synthetic lipid vesicles have been widely used as model membranes in the study of membrane processes (for reviews of liposomes, see/34-36/).

Liposomes can be divided into three classes depending on size and structure:

- 1) Multilayered liposomes consist of several bilayers and vary in size between 0.1 and 10  $\mu\text{m}$ . They are formed by swelling of an aqueous suspension of phospholipids.
- 2) Small bilayer liposomes with only one bilayer and varying in size between 20 and 50 nm. Each liposome contains approximately  $3 \times 10^3$  phospholipid molecules. Small bilayer liposomes are formed by either a)ultra-sonic dispersion, b)injection of an ethanolic solution of phospholipid through a small-bore needle into an aqueous solution, c)passage of an aqueous suspension of phospholipids under high pressure through a French Press or d)solubilizing the phospholipid with the detergent and then removing the detergent by dialysis or gel filtration.
- 3) Large unilamellar liposomes with only one bilayer and ranging in size from 60 nm to several  $\mu\text{m}$ . These

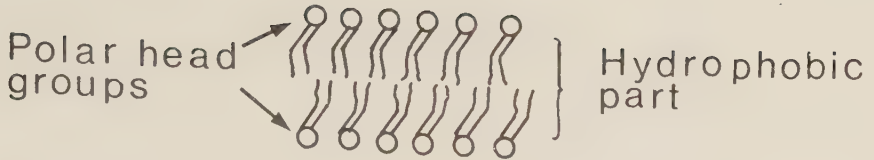


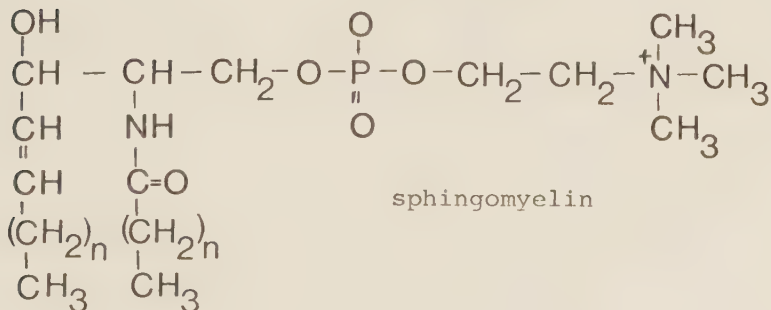
Fig 1. Schematic drawing of a phospholipid bilayer.

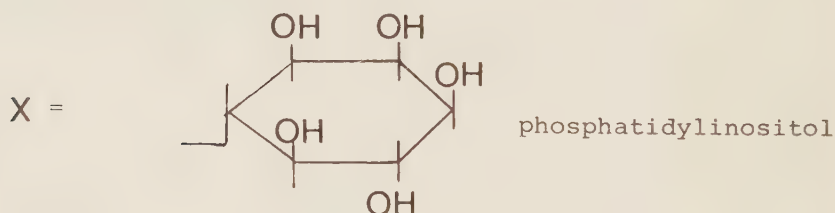
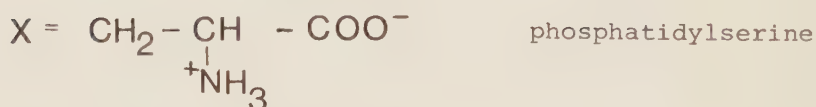
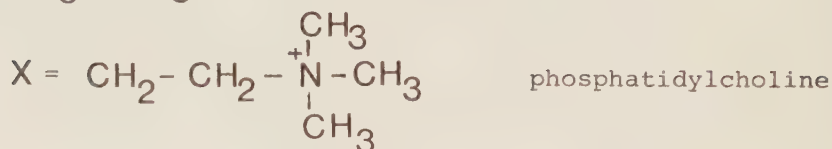
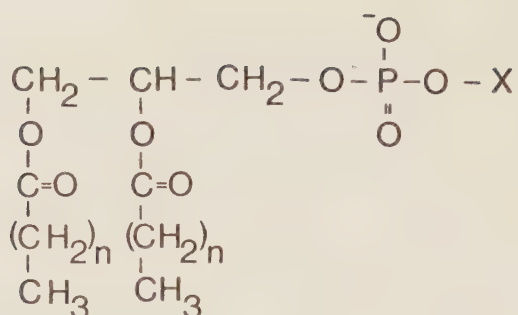
are formed by either a) slowly hydrating a thin layer of phospholipid in distilled water, b) adding  $\text{Ca}^{2+}$  to small phosphatidylserine bilayer liposomes and then adding EDTA, c) injecting an ether solution of lipid into a warm aqueous medium or d) high-speed centrifugation of a water emulsion of lipid in an organic phase into an aqueous phase through an interfacial monolayer of phospholipids.

#### LIPID COMPOSITION.

Liposomes are composed of phospholipids with a hydrophobic part and a polar head group (fig 1).

Formulas of phospholipids:





### FLUIDITY.

Each phospholipid is characterized by a gel-liquid crystalline phase transition temperature  $T_c$  above which its fatty acyl chains are in a more fluid state. In general,  $T_c$  is lowered by decreased chain length and by unsaturation of the acyl chains. The polar head group also influences the transition temperature but to a smaller extent than the acyl chains. Naturally occurring phospholipids (e.g. egg phosphatidylcholine) contain a complex mixture of saturated and unsaturated acyl chains and are characterized by broad low-tempe-

Table II.

T<sub>C</sub>-values of multilayered liposomes.

| Lipid                                                             | T <sub>C</sub> (°C) |
|-------------------------------------------------------------------|---------------------|
| Egg phosphatidylcholine                                           | -15--7              |
| Dilauroyl phosphatidylcholine                                     | ~0                  |
| Dimyristoyl phosphatidylcholine                                   | 23                  |
| Dipalmitoyl phosphatidylcholine                                   | 41                  |
| Distearoyl phosphatidylcholine                                    | 58                  |
| Sphingomyelin (bovine brain)                                      | 32                  |
| Phosphatidylserine (bovine brain)                                 | 5                   |
| Dipalmitoyl phosphatidylcholine :<br>cholesterol, molar ratio 1:1 | none                |

ture transitions (Table II).

#### CHOLESTEROL.

Cholesterol which does not itself form liposomes can be incorporated into liposomal bilayers in amounts up to 50 mol%. Cholesterol broadens the phase transition of phospholipids and may cause it to disappear entirely, as in the case of dipalmitoyl phosphatidylcholine:cholesterol, molar ratio 1:1 (table II). It appears that the presence of cholesterol produces a more condensed state where the hydrocarbon chains of the phospholipid are motionally restricted/35/.



### 3. Aqueous two-phase systems.

Aqueous two-phase systems are obtained by mixing aqueous solutions of the two polymers poly(ethylene glycol) and dextran. The upper phase is rich in poly(ethylene glycol) and the lower phase in dextran. (For reviews of aqueous two-phase systems see /1-5/). Both phases have a high content of water, 80-95%, and are therefore excellent media for biological materials. Salts, sucrose or other water soluble compounds can be included to adjust tonicity, pH or ionic strength.

Particles distribute between the two phases and the interface. Partition of particles is dependent of the molecular weight of the polymers, polymer concentration and temperature. If the molecular weight of one polymer is reduced, the particles will have more affinity for the phase rich in that polymer. Thus if dextran 500,  $M_w=500\ 000$ , is replaced by dextran 40,  $M_w=40\ 000$ , in the dextran-poly(ethylene glycol) phase system, more particles will be found in the lower dextran-rich phase. If the polymer concentration is increased the number of particles adsorbed to the interface will also increase /1/.

#### PHASE DIAGRAMS.

The composition of the phases of a given system can be described in a phase diagram (fig 2) /1/.

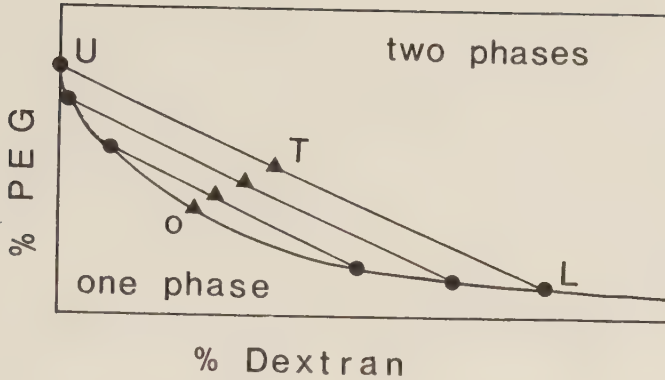


Fig 2. Phase diagram for a dextran-poly(ethylene glycol) phase system.

The curved line is called the binodal curve and represents the minimum polymer concentrations required to obtain two phases. O is the critical point where the compositions and the masses of the two phases become indistinguishable. A phase system of composition T in the phase diagram will have an upper phase composition U and a lower phase composition L. Pairs of points such as U and L are called nodes, and lines joining them are called tie-lines. The mass ratio and, therefore, approximately the volume ratio between the upper and lower phases is equal to the ratio between the distances TL and UT.

The binodal curve and the tie-lines change with temperature and depend on the molecular weights of the polymers. There may also be small variations between polymers of different batches. Changing the polymer concentrations is equivalent to moving from one point

in the phase diagram to another point.

#### SALTS AND INTERFACIAL POTENTIAL.

Partition of particles often depends strongly on the type of salt present in the system /1/. This directing effect of salts on partition increases with the electrical charge of the particle. The effect of the salt on the partition is therefore due to electrical forces. When the anion and the cation of the salt differ in their relative affinity for the phases, a different distribution of the ions will occur at the interface, giving rise to a potential difference between the phases, a so called interfacial potential/37/. If the anion has a greater affinity for the upper phase than the cation, as with NaCl, the upper-phase side of the interface will be negatively and the lower-phase side positively charged.

Conflicting observations concerning the potential created by NaCl have been presented. If the potential was measured by electrodes, no potential difference could be found /22/. If instead the potential was measured by partition of proteins at different pH values a negative potential, i.e. the upper phase more negative than the lower phase, was found /37/. Also in my experience particles behave as if there is a negative potential in phase systems containing NaCl. The partition of DEAE-dextran (positively charged dextran) has been measured in phase systems with Na-phosphate buffer and NaCl in different amounts (table III)/1,38,39/.

Table III.

Partition of DEAE-dextran.<sup>1</sup> Total phase composition was 4% dextran 500, 4% poly(ethylene glycol) 6000, 1.04% DEAE-dextran and the salts indicated.

| Phase  | Salt composition                              | DEAE-dextran<br>partition (mg<br>N per g phase) | Relative<br>phase<br>volume |
|--------|-----------------------------------------------|-------------------------------------------------|-----------------------------|
| Top    | { 0.15 M NaCl + 0.01 M<br>Na-phosphate buffer | 0.472                                           | 0.68                        |
| Bottom |                                               | 0.025                                           | 0.32                        |
| Top    | No salt                                       | 0.339                                           | 0.58                        |
| Bottom |                                               | 0.325                                           | 0.42                        |
| Top    | { 0.11 M Na-phosphate<br>buffer, pH 6.8.      | 0.023                                           | 0.52                        |
| Bottom |                                               | 0.635                                           | 0.48                        |

1) The table is taken from /38/.

When the concentration of NaCl was increased relative to Na-phosphate more DEAE-dextran was found in the upper phase. Thus, increasing concentrations of NaCl seem to create a negative potential. Biological particles, which usually are negative, will in a NaCl system distribute primarily into the lower phase compared to the behaviour in a system with zero interfacial electrical potential. If the salt creates an interfacial potential of the opposite sign, i.e. upper-phase side positively and lower-phase side negatively charged, as with Na-phosphate, the particles can be driven into the upper phase. The charge of the particles can be estimated qualitatively by comparing the effects of two neutral salts on the partition.

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The partition of particles can be strongly influenced by introducing charged groups on a fraction of poly(ethylene glycol)/14,19/. If poly(ethylene glycol) contains positively charged groups, as is the case for trimethylamino poly(ethylene glycol), it will attract negative particles into the upper phase. If, however, particles in an ordinary system are in the upper phase, they can be moved to the interface or to the lower phase by using poly(ethylene glycol) with negatively charged groups, e.g. poly(ethylene glycol)-sulphonate.

When systems with poly(ethylene glycol) of mol.wt. 4000 and at low ionic strength (less than 5 mM) are used, little (less than 5%) of the total poly(ethylene glycol) has to be replaced by its charged form to extract all the material from one phase to the other. Charged poly(ethylene glycol) of mol.wt 6000 has considerably less effect on the partition and a 20-50 times larger concentration is required to obtain the same effect. When charged poly(ethylene glycol) is used for partition, the concentration of other electrolytes in the system must be kept low.

Highly charged dextran, e.g. DEAE-dextran has also been used for partition experiments /38,39/. Because of the very high degree of substitution, these derivatives have lost their dextran-like character, and can be found either in the upper or in the lower phase depending on the electrolytes present (see page 15).



#### HYDROPHOBIC AFFINITY PARTITION.

Fatty acids covalently attached to poly(ethylene glycol) can selectively alter the partition of membrane particles /23,40/. The fatty acid is supposed to dip down into the membrane and in that way anchor the poly(ethylene glycol)-chain to the membrane. The poly(ethylene glycol)-covered membrane surface will force the particle into the upper poly(ethylene glycol)-rich phase. This method has also been used to study hydrophobic interactions between proteins and aliphatic hydrocarbon chains /41/.

The chain-length of the fatty acid esterified to poly(ethylene glycol) is of importance for the partition. Intact and broken chloroplasts have been separated with capric acid attached to poly(ethylene glycol). Shorter or longer fatty acids gave no separation /24/.

## RESULTS.

1 Hydrophobic affinity partition of erythrocytes in aqueous two-phase systems.

Hydrophobic affinity partition of erythrocytes using poly(ethylene glycol) with covalently attached fatty acids or deoxycholic acid resulted in different distribution of erythrocytes from different species/I/. Two groups of erythrocytes were distinguished, one with high affinity for the poly(ethylene glycol)-bound ligand having a high relative amount of phosphatidylcholine compared to sphingomyelin in their membranes. The other group with a low affinity for the poly(ethylene glycol) bound ligand had a higher amount of sphingomyelin relative to phosphatidylcholine.

## SCANNING ELECTRON MICROSCOPY OF ERYTHROCYTES IN AQUEOUS TWO-PHASE SYSTEMS.

There have been reports about the aggregation of erythrocytes in the presence of dextrans of high molecular weight /42/, membrane fusion induced by high concentrations of poly(ethylene glycol), around 50% (w/w), /43,44/ and shape transformation of erythrocytes caused by e.g. fatty acids and lysolecithin /45/. The shape of erythrocytes in phase systems containing poly(ethylene glycol) palmitate was therefore examined by scanning electron microscopy (unpublished).

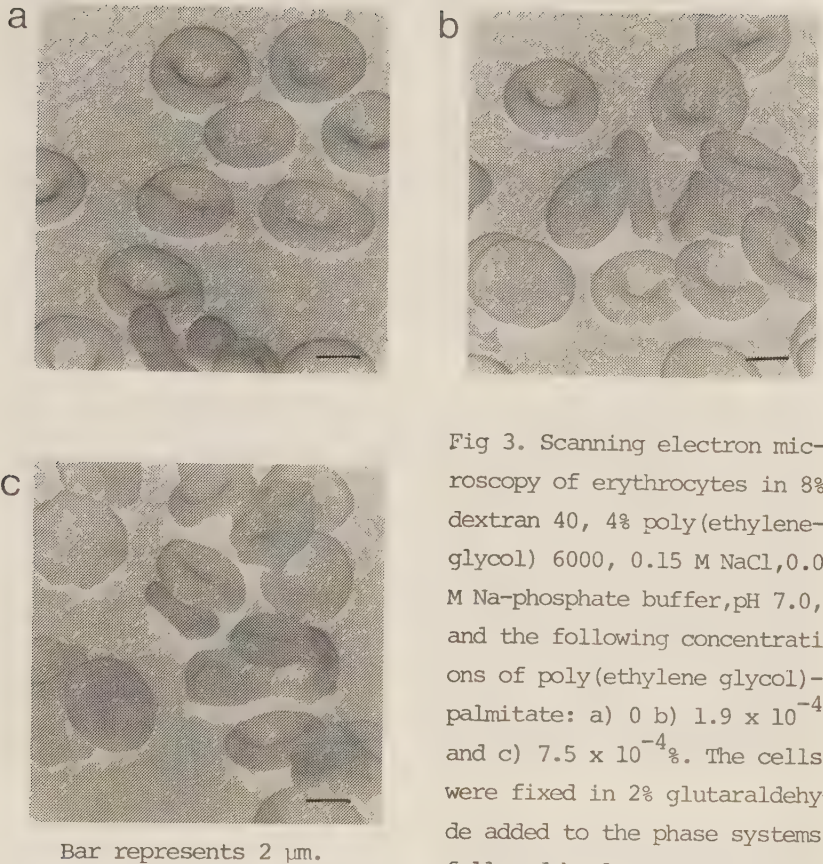


Fig 3. Scanning electron microscopy of erythrocytes in 8% dextran 40, 4% poly(ethylene glycol) 6000, 0.15 M NaCl, 0.01 M Na-phosphate buffer, pH 7.0, and the following concentrations of poly(ethylene glycol)-palmitate: a) 0 b)  $1.9 \times 10^{-4}\%$  and c)  $7.5 \times 10^{-4}\%$ . The cells were fixed in 2% glutaraldehyde added to the phase systems followed by 1%  $\text{OsO}_4$ .

Human erythrocytes partitioned in phase systems containing various amounts of poly(ethylene glycol) palmitate (9.4% substituted) are shown in fig 3. The erythrocytes in fig 3a partition in the lower phase, those in fig 3b collect at the interface and those in fig 3c partition in the upper phase. (Compare fig 2a in /I/ where DS, degree of substitution, is 0, 0.0047% and 0.0188% respectively). The cells in phase systems without poly-

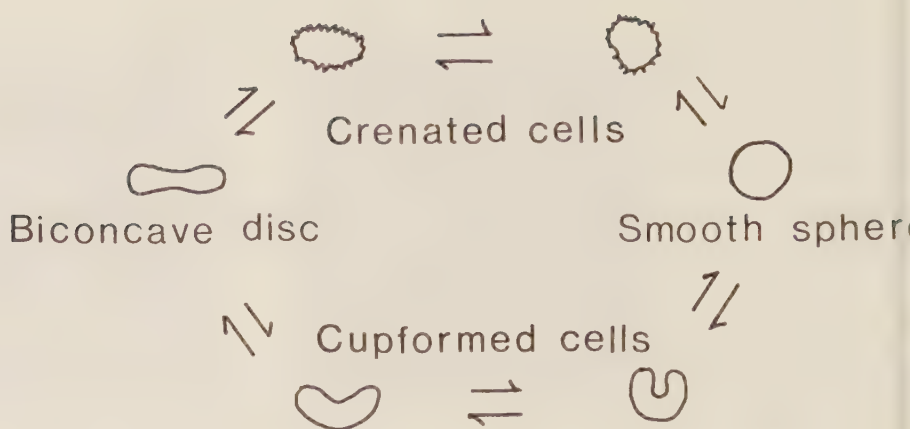


Fig 4. Principal types of shape transformations of human erythrocytes (from /45/).

(ethylene glycol) palmitate have the normal biconcave shape while increasing concentrations of poly(ethylene glycol) palmitate leads to deformed cells.

The biconcave shape of normal human erythrocytes can be altered reversibly by a number of amphiphilic agents, such as fatty acids or lysolecithin /45/(fig 4). A crenation is induced, as a rule, by anionic or non-ionized compounds, and cuplike cells by cationic substances. Both types of transformation are reversible upon removal of the transforming agent.

In order to observe the shape of erythrocytes at even higher concentrations of poly(ethylene glycol) palmitate than in fig 3, scanning electron microscopy pictures were taken at different concentrations of this substituted polymer (fig 5). In this experiment only poly(ethylene glycol) palmitate and salts were present



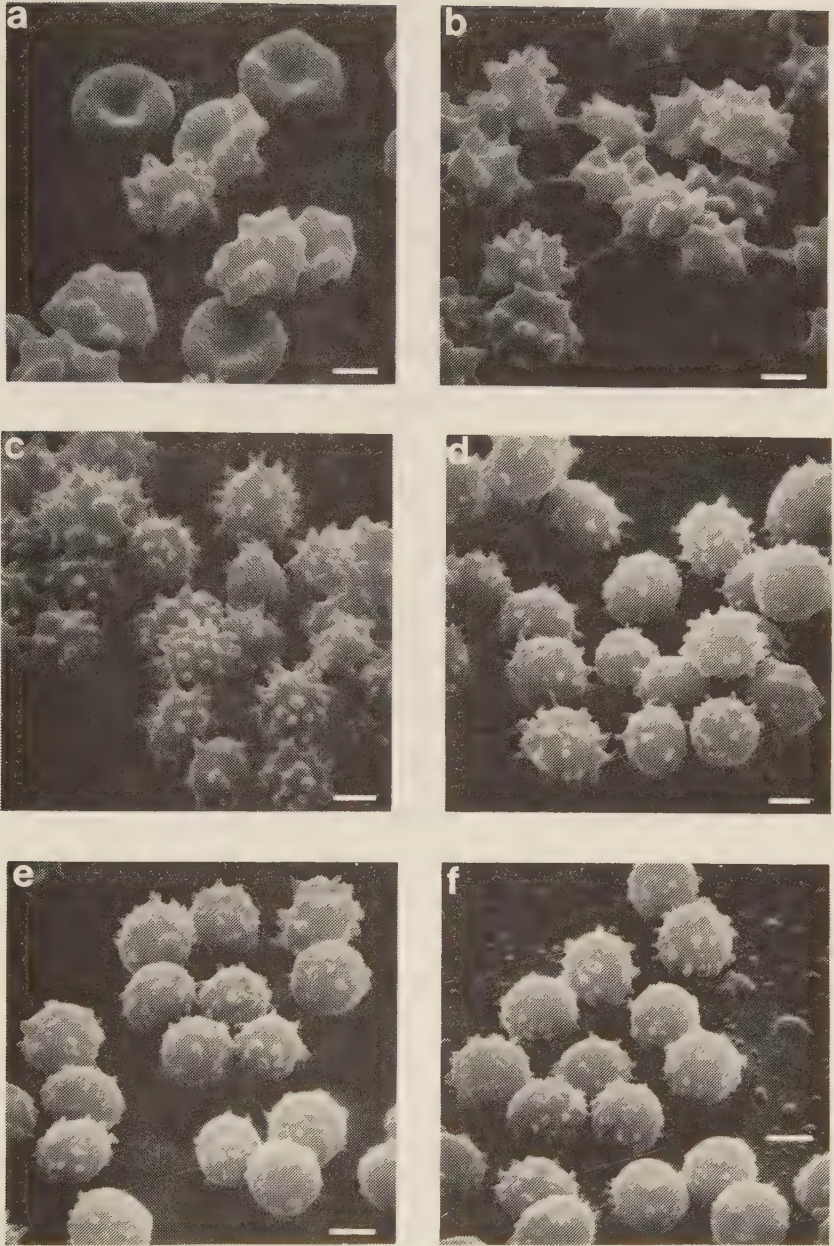


Fig 5. Scanning electron microscopy of erythrocytes in 0.15 M NaCl, 0.01 M Na-phosphate buffer, pH 7.0, and poly(ethylene glycol) palmitate (13.6% substituted) in the following concentrations: a)  $1.4 \times 10^{-4}\%$ ; b)  $4.1 \times 10^{-4}\%$ ; c)  $1.4 \times 10^{-3}\%$ ; d)  $4.1 \times 10^{-3}\%$ ; e)  $1.4 \times 10^{-2}\%$  and f)  $4.1 \times 10^{-2}\%$ . Bar represents 2  $\mu\text{m}$ .



(no phase systems). For a comparison with fig 1 in /I/ the concentrations of poly(ethylene glycol) palmitate in fig 5a-f correspond to log DS values of -4.5, -3.9, -3.5, -2.9, -2.5 and -1.9 respectively.

There was a protective effect of the phase systems as can be seen by comparing the erythrocytes in figs 3b and 5a and in figs 3c and 5b. The erythrocytes exposed to poly(ethylene glycol) palmitate in the presence of phase systems are less crenated than cells exposed to poly(ethylene glycol) palmitate only. Higher concentrations of poly(ethylene glycol) palmitate will give progressively more crenated erythrocytes.

Besides the aggregation of erythrocytes caused by highly substituted poly(ethylene glycol)/I/, some aggregation was observed with poly(ethylene glycol) of low degree of substitution (cf fig 5 b and c). It appears that the erythrocytes aggregate more easily at some stages in the crenation process.

## 2. Partition of liposomes in aqueous two-phase systems.

### 2.1 Are liposomes a valid model system for partition of membranes in two-phase systems?

The question is whether liposomes are affected in the same way as membranes by alterations in the phase systems. E.g. alterations in molecular weight of the polymers, polymer concentration, salts or poly(ethylene glycol) with charged or hydrophobic groups.

Liposomes distribute between the two phases and the interface. Small unilamellar liposomes and large multilayered liposomes differ in their distribution. Larger liposomes collect more at the interface than unilamellar liposomes (cf table I in /II/). Adsorption at the interface has been reported to occur more frequently for larger particles than for smaller ones /1/.

### MOLECULAR WEIGHTS OF THE POLYMERS.

If dextran 500 is replaced by dextran 40 in the poly(ethylene glycol)-dextran phase system, more liposomes will be found in the dextran-rich phase /II/. This is a general effect which has been shown both with particles and proteins /1/.

## POLYMER CONCENTRATION.

In a phase system consisting of 5.6% dextran 500, 5.6% poly(ethylene glycol) 4000, 5 mM NaCl, 10 mM Na-phosphate buffer, pH 7.4, and 20 mM sucrose at 4°C more than 90% of egg phosphatidylcholine liposomes partition in the upper phase. If the polymer concentration is increased to 7.0% dextran 500 and 7.0% poly(ethylene glycol) 4000 there is an increased adsorption of liposomes at the interface (fig 6).

Both multilamellar and bilayer phosphatidylserine liposomes have a lower partition in 5.6% dextran 500 and 5.6% poly(ethylene glycol) 4000 (same salts as above) than phosphatidylcholine liposomes. More than 80% of multilamellar phosphatidylserine liposomes are in the lower phase while remaining ones are at the interface. When the polymer concentration is increased to 7.0% dextran 500 and 7.0% poly(ethylene glycol) 4000 more liposomes will adsorb to the interface.

Partition of mixed liposomes composed of both phosphatidylserine and phosphatidylcholine also results in increasing amounts at the interface as the polymer concentrations increase. Thus, adsorption at the interface increases as the polymer concentrations increase, regardless of whether liposomes mostly are in the lower or upper phase at low polymer concentrations (fig 6).

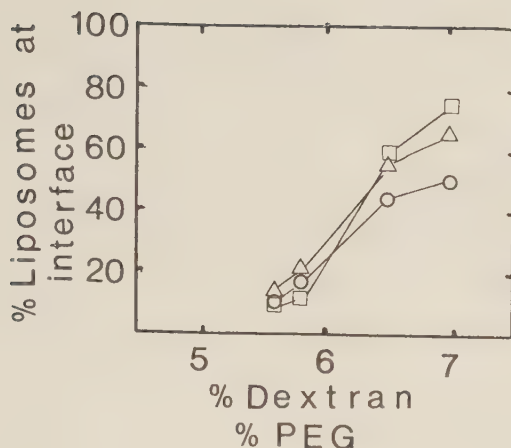


Fig 6. The percentage of bilayer liposomes at the interface as function of the percentage of dextran and poly(ethylene glycol) in the phase systems.  $\Delta$ , phosphatidylserine;  $\square$ , egg phosphatidylcholine and  $\circ$ , egg phosphatidylcholine:phosphatidylserine in a molar ratio of 1:1.

#### SALTS.

Negatively charged liposomes have a lower partition in phase systems with a negative potential (the upper phase more negative than the lower phase) than in systems with a positive one /II/. Liposomes with no net charge but with one positive and one negative charge on each lipid (for instance phosphatidylcholine and sphingomyelin) have a slightly but significantly higher partition in phase systems with a negative potential compared to a positive one, i.e. they behave as if they carry a positive surface charge. The reason for this might be that the positive charge is further out from the liposome surface than the negative charge and that the phase system can detect this dipolar charge.

## POLY(ETHYLENE GLYCOL) WITH CHARGED GROUPS.

The partition of negatively charged phosphatidylserine liposomes can be changed from the lower phase to the upper phase by including positively charged trimethylamino poly(ethylene glycol) in 5.8% dextran 500, 5.8% poly(ethylene glycol) 4000 and 1.1 mM NaCl at 4°C. Egg phosphatidylcholine liposomes partition in the upper phase in this system and the distribution is not affected by the addition of trimethylamino poly(ethylene glycol) (unpublished). Poly(ethylene glycol) with a charge of the opposite sign as the partitioned particles is supposed to attract the particles to the upper phase, while poly(ethylene glycol) with a charge of the same sign is supposed to repel the particles. Thus, the weakly positive charge of egg phosphatidylcholine found by partition in phase systems with different salts /1/ was not detected with trimethylamino poly(ethylene glycol).

The ionic strength has to be very low in phase systems utilizing trimethylamino poly(ethylene glycol)/5/. A positive charge of phosphatidylcholine liposomes was detected in phase systems containing 150 mM NaCl but not in 1.1 mM NaCl. A conformational change of the polar head groups of phosphatidylcholine liposomes have been suggested to occur as the NaCl concentration increases /46/. In this model the positive charge of phosphatidylcholine is protruding from the liposomal surface at higher NaCl concentrations. This would explain the



positive charge observed in high-salt phase systems, but not in low-salt phase systems with trimethylamino poly(ethylene glycol).

#### *POLY(ETHYLENE GLYCOL) WITH HYDROPHOBIC GROUPS.*

Fatty acids covalently bound to poly(ethylene glycol) change the partition of liposomes from the lower phase to the upper phase /II,III/. The hydrocarbon chains dip down into the membrane and interact with the hydrophobic interior of the bilayer. For bilayer vesicles the amount of poly(ethylene glycol) palmitate that is required to obtain 50% of the liposomes in the upper phase is in the order of 5 poly(ethylene glycol) palmitate molecules per liposome. The corresponding number for erythrocytes was  $5 \times 10^6$  molecules per erythrocyte (fig 7). The amount of poly(ethylene glycol) palmitate per mole of membrane lipid required to withdraw the particles into the upper phase is 10 times higher for erythrocytes than for bilayer liposomes. It is possible that the proteins of the erythrocyte

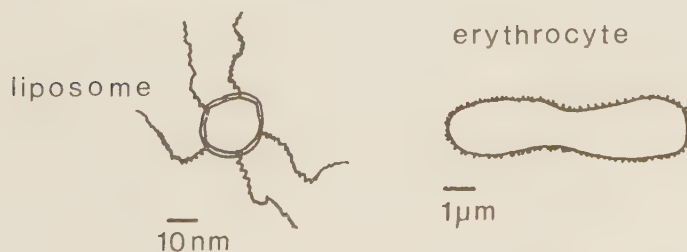


Fig 7. Schematic drawing of a liposome and an erythrocyte with protruding poly(ethylene glycol) palmitate molecules.

membrane are a hindrance for the interaction between poly(ethylene glycol) palmitate and the hydrophobic part of the membrane.

Two groups of erythrocytes differing in their content of sphingomyelin and phosphatidylcholine could be distinguished using poly(ethylene glycol) palmitate in the phase system /I/. This finding could be verified with liposomes /II/.

In summary, liposomes behave very similarly to membranes in phase systems and they are therefore a valid model system for studying the factors determining the partition of membrane particles in aqueous two-phase systems.

## 2.2 Factors that determine the partition of liposomes.

How deeply embedded membrane differences can be detected by phase systems? To examine this question the partition behaviour of liposomes with variations at different levels of the bilayer has been studied /II,III/.

### THE HYDROPHOBIC PART OF THE MEMBRANE.

Variations in the hydrophobic part of the membrane were investigated using phosphatidylcholine liposomes with different degrees of unsaturation in the phospholipid fatty acyl chains. Soya bean phosphatidylcholine was the most unsaturated phospholipid used, egg phosphatidylcholine was intermediate and dipalmitoyl phosphatidylcholine was entirely saturated.

There were no differences in partition of multilayered liposomes with different degrees of unsaturation. In contrast, small bilayer liposomes prepared from soya bean phosphatidylcholine had a higher partition than liposomes with more saturated acyl chains. Since bilayer liposomes have a higher radius of curvature than multilayered liposomes, the hydrophobic part of the membrane may be more exposed in the case with bilayer vesicles. This may explain why the degree of unsaturation is reflected in partition of bilayer liposomes but not in partition of multilayered vesicles.

Incorporation of cholesterol into small liposomes of soya bean phosphatidylcholine and dipalmitoyl phosphatidylcholine equalizes the partition of these lipo-

somes. Since most membrane vesicles are of approximately the same size as multilayered liposomes (0.1-10  $\mu\text{m}$ ) and often contain cholesterol, it seems that the degree of unsaturation of the phospholipid acyl chains does not influence the partition.

CAN DIFFERENCES IN THE HYDROPHOBIC PART OF THE BILAYER BE DETECTED WITH A HYDROPHOBIC LIGAND ATTACHED TO POLY(ETHYLENE GLYCOL)?

A hydrophobic ligand attached to poly(ethylene glycol) can change the partition of liposomes from the lower phase via the interface to the upper phase. The hydrophobic ligands used here have been fatty acids varying in length from 10-18 carbon atoms /III/. Liposomes prepared from synthetic phosphatidylcholine with saturated fatty acids varying in length from 12-18 carbon atoms and unsaturated egg phosphatidylcholine liposomes were partitioned in phase systems containing the poly(ethylene glycol)-bound fatty acids. There were no differences in partition in these phase systems between the different phosphatidylcholine liposomes. All phosphatidylcholine liposomes were removed from the lower phase at the same concentration of each poly(ethylene glycol)-ligand examined. The results show that the hydrophobic interior of the bilayer is of minor importance for the partition even in phase systems with hydrophobic ligands attached to poly(ethylene glycol).

A correlation between the composition of the hyd-

rophobic part of the membrane and the partition in two-phase systems have been reported. Thus, Walter et al /22/ found a correlation between the ratio of poly-/mono-unsaturated fatty acids in the membrane and the partition of erythrocytes. This finding could not be verified with liposomes. However, each phospholipid in the erythrocyte membrane is characterized by a specific fatty acyl chain composition /47/. Thus, there is a connection between the kind of phospholipid and its fatty acid composition. This means that a correlation to the degree of unsaturation would also be a correlation to the polar head groups of the phospholipids.

#### THE INTERMEDIATE PART OF THE BILAYER BETWEEN THE HYDROPHOBIC PART AND THE POLAR HEAD GROUPS.

In phase systems where no ligand is attached to poly(ethylene glycol), sphingomyelin and phosphatidylcholine liposomes have a similar distribution /II/. In phase systems containing palmitic acid esterified to poly(ethylene glycol) a difference in partition between these liposomes can be seen. Higher concentrations of poly(ethylene glycol) palmitate are required to obtain sphingomyelin liposomes in the upper phase compared to phosphatidylcholine liposomes. This is in line with the results in /I/ where erythrocytes with a high sphingomyelin to phosphatidylcholine ratio required higher concentrations of poly(ethylene glycol)-ligands to collect in the upper phase.

The fact that sphingomyelin-rich membranes require



higher concentrations of poly(ethylene glycol) palmitate than phosphatidylcholine-rich membranes for extraction into the upper phase may be explained by considering the "hydrogen belts" of the membranes /48/. These regions consist of hydrogen bond acceptors (e.g. carbonyl groups of glycerol- and sphingo-lipids) and hydrogen bond donors (e.g. sphingolipid hydroxyl groups). In the case of sphingomyelin liposomes there are several opportunities for lipid-lipid hydrogen binding, since sphingomyelin contains both donor and acceptor. In contrast, phosphatidylcholine only contains acceptor. Thus, there is likely to be a network of hydrogen bonds in sphingomyelin liposomes which might be difficult to penetrate for poly(ethylene glycol) palmitate molecules.

#### THE POLAR HEAD GROUPS.

Liposomes composed of phospholipids with different polar head groups have been partitioned in phase systems /II,III/. Large differences in partition of liposomes were found when the polar head groups were varied. The charge of a liposome is important for its partition behaviour. It is not only the charge that is of importance however. For instance phosphatidylserine and phosphatidylinositol liposomes have the same net charge, but not the same partition behaviour. A further example is the partition of liposomes composed of phospholipids where the methyl groups of phosphatidylcholine have been substituted with ethyl groups (fig 8, unpublished).

# Variations in polar head groups

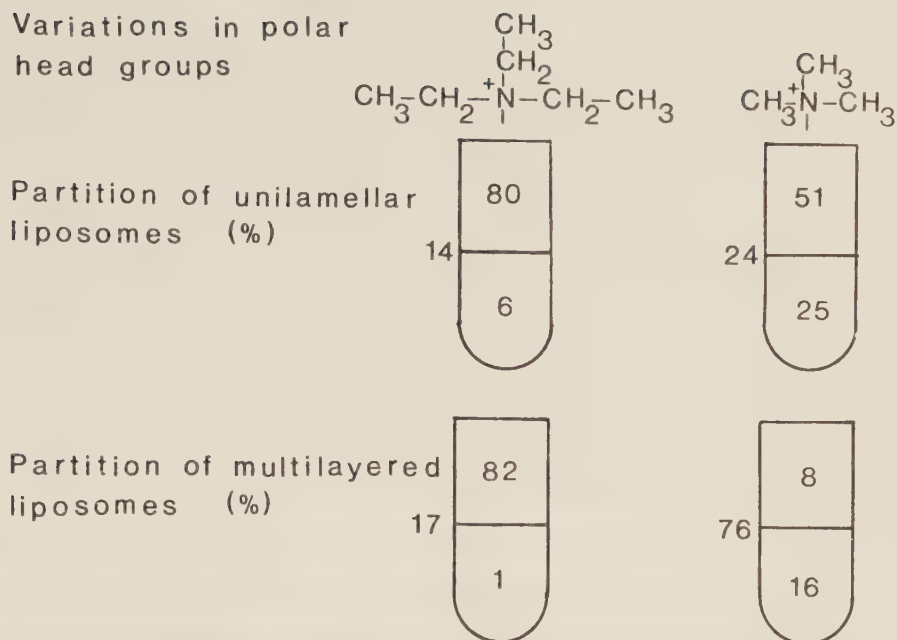


Fig 8. Partition of liposomes composed of soya bean phosphatidylcholine and its modified analogue in 5% dextran 500, 4% poly(ethylene glycol) 6000, 0.15 M NaCl and 0.01 M Na-phosphate buffer, pH 7.0. The modified phospholipid was a generous gift from Dr Björn Åkesson.

The exchange of three methyl groups to three ethyl groups results in a higher partition of both unilamellar and multilayered liposomes.

Phosphatidylserine liposomes favour the dextran-rich phase more than phosphatidylinositol liposomes (table I in /II/). This was a somewhat surprising finding since there is a structural resemblance between the polar head group of phosphatidylinositol and the dextran molecules (both contain carbohydrate).

In phase systems containing poly(ethylene glycol)-bound fatty acids the selectivity in partition of liposomes is determined by the polar head groups of the phospholipids. Although the presence of the hydrophobic part of the bilayer is necessary for this hydrophobic affinity partition of liposomes, the structure of the hydrophobic part is not important for the partition.

#### PARTITION OF MIXED LIPOSOMES.

Results from partition experiments using liposomes composed of mixed phospholipids show that no linear relationship exists between the partition and the amount of each phospholipid. A mixture of phosphatidylserine and phosphatidylcholine liposomes had a higher partition than liposomes composed of each individual phospholipid (table I in /II/). This is an illustration of the difficulty in predicting the partition of a membrane with known lipid composition.

#### 2.3 Incorporation of pigments and cholesterol into the liposomal bilayer.

The effect of incorporation of molecules such as pigments and cholesterol on the partition of liposomes has been studied.

#### PIGMENTS.

Chlorophyll a, chlorophyll b and  $\beta$ -carotene were cosonicated with egg phosphatidylcholine or phosphatidylserine. The liposomes obtained were partitioned in

5.6% dextran 500; 5.6% poly(ethylene glycol) 4000, 5 mM NaCl, 10 mM Na-phosphate buffer, pH 7.4, and 20 mM sucrose at 4°C (unpublished results). In this phase system both multilamellar and bilayer egg phosphatidylcholine liposomes favour the upper phase to more than 90%, more than 80% of multilayered phosphatidylserine liposomes partition into the lower phase, while bilayer phosphatidylserine liposomes have an intermediate partition. No effect on the partition could be found after incorporation of chlorophyll a, chlorophyll b or  $\beta$ -carotene into these liposomes. Not even in molar ratios of 5.6:1, phospholipid:pigment. Chlorophyll incorporated into liposomal bilayers appears to have the porphyrin ring located in the polar head group region of the bilayer and the phytol chain penetrating the hydrophobic core /49/(fig 9).  $\beta$ -carotene is supposed to be located in the hydrophobic portion of the membrane /50/(fig 9).

#### CHOLESTEROL.

Cholesterol was incorporated into liposomes in a molar ratio of 1:1 with phosphatidylcholine molecules of different degrees of unsaturation or with sphingomyelin /II/. Partition differences between bilayer liposomes composed of soya bean phosphatidylcholine or dipalmitoyl phosphatidylcholine were reduced in the presence of cholesterol. Incorporation of cholesterol into phospholipid membranes produces restriction of molecular motion in the region of the first eight to

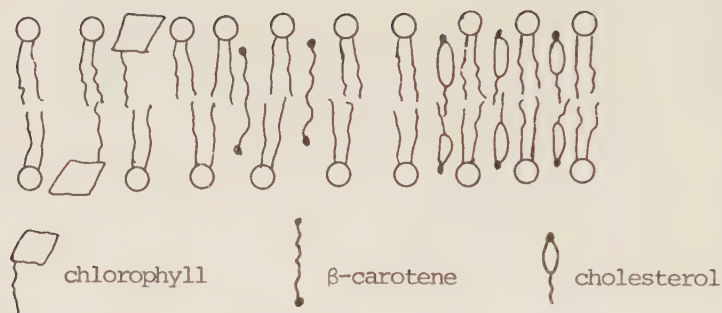


Fig 9. The orientation of chlorophyll,  $\beta$ -carotene and cholesterol in a phospholipid bilayer.

ten carbon atoms of the acyl chains from the lipid-water interface. Since bilayer liposomes with a high radius of curvature may expose some hydrophobic parts of the bilayer, the addition of cholesterol would have an equalizing effect on the exposed parts. This may explain the equalizing effect of cholesterol on the partition of liposomes with different degrees of unsaturation.

The positive surface charge of phosphatidylcholine and sphingomyelin liposomes that was detected by the phase systems was increased when cholesterol was incorporated into the liposomal membranes. Thus, the presence of cholesterol seems to further add to the protrusion of the positive charge.

In phase systems containing poly(ethylene glycol) palmitate the incorporation of cholesterol into multilayered phosphatidylcholine liposomes had no effect on the partition behaviour. Bilayer liposomes prepared



from phosphatidylcholine and cholesterol require slightly smaller concentrations of poly(ethylene glycol) palmitate for extraction into the upper phase than liposomes without cholesterol.

However, the alterations in partition of liposomes observed after incorporation of cholesterol are only of limited importance and the conclusion must be that cholesterol plays a minor role in determining the partition of liposomes.

### 3. The effect of proteins on the partition of membranes.

The effect of trypsin or neuraminidase treatment on the partition of erythrocytes in two-phase systems has been studied by Walter et al /51/. Such a treatment leads to a release of sialic acid which is the main contributor to the negative charge of the proteins in the erythrocyte membrane. In phase systems with positive potentials, i.e. the upper phase more positive than the lower phase, erythrocytes with a high content of sialic acid were found in the upper phase, but not erythrocytes of which the sialic acid was removed /51/. Thus, the removal of the negatively charged proteins on the erythrocyte surface causes an alteration in partition of erythrocytes in aqueous two-phase systems.

Partition experiments have also been performed with lipoproteins in two-phase systems (unpublished).

High density lipoproteins have a surface layer consisting of phospholipids, cholesterol and proteins in the approximate ratio of 23:20:55. The major phospholipids are phosphatidylcholine and sphingomyelin. High density lipoproteins appear to be spherical and range in size from 7-12 nm. In phase systems composed of 5% dextran 500, 4% poly(ethylene glycol) 6000, 0.15 M NaCl and 0.01 M Na-phosphate buffer, pH 7.0, 27% of the high density lipoproteins are found in the upper phase, 1% collect at the interface and 72% are found in the lower phase. This distribution differs markedly from the partition of bilayer phosphatidylcholine or sphingomyelin liposomes with or without cholesterol (table I in /II/). The proteins, therefore, seem to be of importance for the partition also in this case.

Thus, it is reasonable to assume that proteins influence the partition of membranes. Since different proteins differ in their distribution in aqueous two-phase systems /1/, each protein probably contributes to the partition in its particular manner.

## SUMMARY.

Partition of erythrocytes from different species in aqueous two-phase systems showed a correlation between the partition behaviour and the relative amount of phosphatidylcholine and sphingomyelin in the erythrocyte membrane. This correlation was also seen in the partition of phosphatidylcholine and sphingomyelin liposomes.

The factors that determine the partition of liposomes has been summarized in fig 10.

By partition in two-phase systems containing fatty acids covalently bound to poly(ethylene glycol), the specificity for membranes is improved. Only a small amount of poly(ethylene glycol)-bound fatty acid is required for extraction of membranes from the lower phase

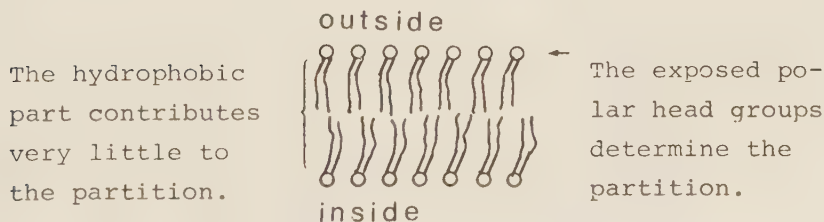


Fig 10. Factors that determine the partition of liposomes in aqueous two-phase systems.

to the interface and the upper phase. In this way membranes are easily separated from non-membrane particles. In phase systems containing poly(ethylene glycol) palmitate the selectivity in partition of liposomes is determined by the polar head groups. The presence of the hydrophobic part of the bilayer is necessary for this hydrophobic affinity partition, but neither the degree of unsaturation nor the length of the phospholipid fatty acids influences the partition behaviour. Some possible kinds of interactions between poly(ethylene glycol)-bound fatty acids and the lipid bilayer are shown in fig 11. The partition depends on the length of the poly(ethylene glycol)-bound fatty acid and on the liposomal polar head groups.

If molecules, such as pigments or cholesterol, are incorporated into the liposomal bilayer, no effect is seen on the partition in two-phase systems.

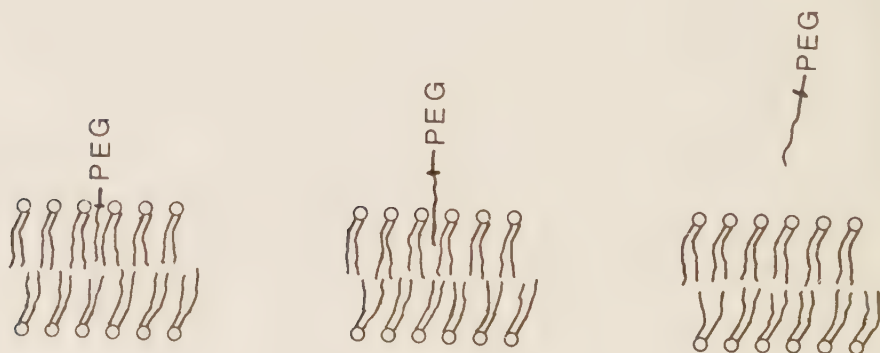


Fig 11. Poly(ethylene glycol)-bound fatty acid interacting with a liposomal bilayer.

The fact that there is no linear relationship between the partition and the composition of mixed liposomes makes it difficult to predict the partition of a membrane with known chemical composition. Instead of predicting the partition, the best way is to partition the membrane in any phase system with a salt composition suitable for the membrane. If the distribution of the membrane is unsatisfactorily in the first phase system chosen, changes in phase- or salt-composition usually helps. Some of these changes are listed below as a guideline for a new partitioner:

- 1) A change of salt which gives another interfacial potential (cf p.14)
- 2) A change in the mol.wt of the polymers (cf p.12).
- 3) A change in the polymer concentration (cf p.12).
- 4) Use polymers with charged or hydrophobic ligands (cf p.16,17)
- 5) Use a multistep procedure such as counter-current distribution.

In this way it is possible to separate and/or study almost all membranes successfully.

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Ett varmt tack till Mats som skött om Daniel och hemmet medan denna avhandling har färdigställts.

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## HYDROPHOBIC SURFACE PROPERTIES OF ERYTHROCYTES STUDIED BY AFFINITY PARTITION IN AQUEOUS TWO-PHASE SYSTEMS

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### Summary

Erythrocytes from various species have been partitioned in aqueous two-phase systems consisting of water, dextran, poly(ethylene glycol), salt and buffer. The terminal hydroxyl groups of the latter polymer were esterified with palmitic, oleic, linoleic and linolenic acids, as well as with deoxycholic acid. In a two-phase system containing unesterified poly(ethylene glycol) the erythrocytes are exclusively in the dextran-rich lower phase. When the poly(ethylene glycol) is esterified the red blood cells collect at the interface and/or in the poly(ethylene glycol)-rich upper phase depending on the type and concentration of esterified acid. Palmitate ester is most effective in increasing the affinity of the cells for the upper phase, followed by oleate, linolate, linolenate, and deoxycholate esters. The partition behaviour of erythrocytes from various species differs considerably. Two groups can be distinguished: one consisting of erythrocytes from dog, guinea pig and rat, the other from human, sheep and rabbit. This division can be correlated to the content of sphingomyelin and phosphatidyl choline in the erythrocyte membranes.

### Introduction

Selective partition of proteins<sup>1,2,3</sup> and membranes<sup>4</sup> in aqueous polymer two-phase systems in which a specific ligand has been covalently

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attached to one of the polymers has recently been demonstrated in this and other laboratories. The name affinity partition is used for this technique in analogy with affinity chromatography.

In this communication we describe the application of this technique on whole cells viz. red blood cells from different species. We utilized the affinity of the erythrocyte membrane for hydrophobic substances. Fatty acids and deoxycholic acid were therefore esterified with poly(ethylene glycol) via its hydroxyl end-groups. The esters partition like poly(ethylene glycol) i.e. they are concentrated in the upper phase of a dextranpoly(ethylene glycol) system.<sup>5</sup> Polymers carrying such hydrophobic groups have a very strong influence on the partition of red blood cells. The effect of various esters on different species were studied in order to correlate this with the lipid composition of the red cell membrane. It is suggested from our studies that partition in two-phase systems can be used to investigate the interaction between membrane surfaces and hydrophobic groups.

### Materials and Methods

Dextran T 40 batch No 4743,  $M_w = 42000$ , was supplied by Pharmacia, Uppsala, Sweden. Poly(ethylene glycol),  $M_n = 6000$ , was obtained from Union Carbide, New York as Carbowax. Poly(ethylene glycol) is abbreviated PEG.

*Synthesis of PEG-esters.* PEG was reacted with dicyclohexylcarbodiimide according to Vowinkel<sup>6</sup>. 1.2 kg of PEG was dissolved in 9.5 l. of

toluene. About 2 l. toluene were distilled off to remove traces of water from the solution. After addition of 84 g of dicyclohexylcarbodiimide (Fluka, Switzerland) and 0.4 g CuI the mixture was mechanically stirred and kept at 40 °C for 22 h in a closed vessel. The iodide was removed by suction filtration and the solution was filtered through a bed of 100 g of aluminium oxide (Matheson, Colman & Bell, Ohio). The filtrate contained 133 g/l. of "activated" PEG. To 375 ml of this solution was added 14 mequi. of oleic, linoleic or linolenic acid. The mixture was kept at 40 °C in a closed vessel for 48 h. The crystals formed were removed by filtration and the polymer precipitated at 3 °C. It was collected by suction and dissolved in 500 ml absolute ethanol and a light stream of H<sub>2</sub>S was bubbled through the solution during 1 min. 5 g of aluminium oxide was added and the solution was filtered and the polymer was precipitated in the cold. It was recrystallized twice from 500 ml absolute ethanol and each time washed with the same amount of this solvent. The polymer was melted and traces of solvent were evaporated (rotational evaporator) in vacuum at 80 °C. The yield was 60–70% calculated on PEG. The esters of unsaturated fatty acids were analyzed by determining their capacity to bind iodine bromide<sup>7</sup>. 2 g polymer was dissolved in 15 ml concentrated acetic acid. 10 ml 0.4% (w/w) IBr was added and after 30 min in darkness at room temperature 10 ml aqueous 2% NaI was added. The solution was rapidly titrated with 0.05 M Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. The endpoint of the titration was easily detectable by the colour change from yellow to colourless. The degree of substitution was 11% for oleate, 6% for linolate and 6.5% for linolenate when calculated on a molecular weight of 5000 for PEG. PEG deoxycholate was prepared similarly, but with a tenfold amount of deoxycholic acid. The degree of substitution, 8%, was determined polarimetrically by comparing the optical activity of aqueous solutions of PEG deoxycholate and of sodium deoxycholate at 589 nm. PEG palmitate, not fully substituted was prepared via palmitoyl chloride as has been described earlier<sup>1</sup>. By using an excess of palmitoyl chloride, fully esterified PEG could also be obtained. The degree of substitution was determined by saponification of the polymer (dissolved in 10 ml of water free

from carbonic acid) with 10 ml 50 mM NaOH at room temperature during 7 days, followed by titration with 0.5 M HCl to pH 8.5. 60% of the end groups of PEG were esterified, calculated on a molecular weight of PEG of 6000.

*Erythrocytes.* The blood cells were kept in a heparin solution at 4 °C. Before use they were washed three times with 0.15 M NaCl and centrifuged at 5' × 1000 g between the washings. The erythrocytes were then suspended in 0.15 M NaCl in order to make a stock suspension of erythrocytes. This was made such that when 1 ml of the stock suspension was centrifuged and the pellet lysed with 10 ml water an absorbance of 0.5 at 540 nm was obtained.

*Phase systems and partition.* The phase systems used contained 4% (w/w) PEG including the esterified PEG and 8% (w/w) dextran T 40. Phase systems of total weight 5 g were prepared by mixing 2 g of a 20% (w/w) dextran solution with 0.5 g of a 40% (w/w) PEG solution (including PEG derivative) and then adding NaCl and sodium phosphate buffer, pH 7.0, to final concentrations of 0.15 M and 0.01 M respectively. 1 ml of the erythrocyte suspension was included in these 5 g. The phases were mixed by 30 inversions and then allowed to separate for 25 min. After separation 1 ml of the upper phase was sucked off with a pipette, washed with 0.15 M NaCl and centrifuged 5' × 1000 g. The pellet was lysed and diluted with 3 ml distilled water and the absorbance at 540 nm was determined.

The percentage of erythrocytes in the upper phase was calculated from the absorbance values and the volume of the upper phase (2.05 ml). All experiments were carried out at room temperature.

## Results

When erythrocytes are partitioned in the PEG-dextran-water two-phase system the cells are found exclusively in the lower phase. When a part of the PEG is replaced by PEG-ester the erythrocytes start collecting at the interface and at higher concentrations of the ester they are



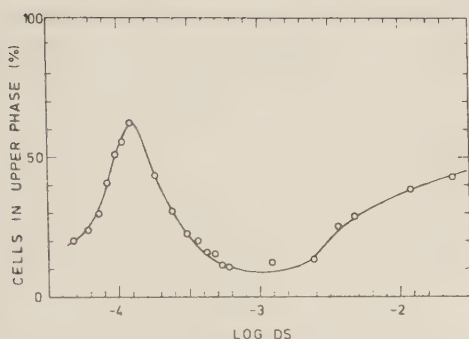


FIG. 1. The percentage of sheep erythrocytes in the upper phase as function of the fraction of PEG end groups esterified with palmitate, DS (degree of substitution).

increasingly transferred to the upper phase. In the case of PEG palmitate further increase in concentration of ester causes conglomeration of the cells. The conglomerates collect mostly at the interface. At still higher concentrations of PEG palmitate the cells do not aggregate and are found in the upper phase. This is shown in Figure 1 where the amount of red blood cells in the upper phase is plotted versus the concentration of PEG palmitate. The aggregation also appears when PEG palmitate alone is added to a saline suspension of erythrocytes in the same concentration as in the two-phase systems. Even here there is deaggregation at higher concentration of PEG palmitate and under microscope the cells seem to have a rounder shape and their surface looks smoother. This phenomenon was not observed when the other PEG-esters were used.

Erythrocytes from sheep were lysed if the concentration of PEG linolate, PEG linolenate or PEG deoxycholate exceeded 1.5%. No such effect was found with PEG palmitate or PEG oleate. In Figure 2 is shown how erythrocytes from various species increase their affinity for the upper phase when the concentration of PEG-ester is increased (below the point where aggregation occurs). The erythrocytes fall into two groups, independent of the acid esterified with PEG; one in which the cells are extracted by the upper phase at low concentration of the ester (rat, dog and guinea pig) and the other in which this occurs at somewhat higher concentration (rabbit, sheep and human). This division is

most conspicuous in the case of PEG linolenate, Figure 2d. The order in which the cells are extracted within a group varies with the esters. The effectiveness of the esters for the first group is in the following order; PEG palmitate: guinea pig < rat < dog; PEG oleate: rat < dog = guinea pig; PEG linolate: dog = guinea pig < rat; PEG linolenate: dog = rat = guinea pig; PEG deoxycholate: rat < guinea pig < dog.

The partition of erythrocytes is affected even when a minute amount of PEG in the system is esterified. Thus, in the most extreme case only 0.005% of the hydroxyl groups of PEG have to be esterified to get 50% of the cells in the upper phase; results with dog erythrocytes and PEG palmitate, Figure 2a. The effectiveness decreases with increasing unsaturation of the esterified acid and higher concentration of ester is therefore necessary to influence the partition. PEG palmitate is on an average 1.8 times more efficient than PEG oleate and 6 times more effective than PEG linolate or PEG linolenate. PEG deoxycholate is 2500 times less effective than PEG palmitate.

The partition is dependent on the amount of erythrocytes in the system, Figure 3. When the number of cells increases their *relative* amount in the upper phase decreases while the *absolute* concentration in the upper phase is nearly constant. This concentration dependence indicates a direct binding between the cells and the PEG-ester. The comparison between different species was carried out at the same concentration... of red blood cells in order to avoid differences due to concentration effects.

The binding of PEG palmitate to the erythrocytes seems to be reversible since the effect of the ester vanished when the cells were washed with saline and repartitioned in an ester-free system.

## Discussion

The effect of the PEG-esters on the partition of erythrocytes can be explained by assuming a binding of the (PEG-bound) fatty acid respectively deoxycholic acid to the cell surface. Consequently, the higher the content of PEG-ester in the systems the larger is the part of the cell surface covered with PEG-chains. In an ester-free system the blood cells prefer the

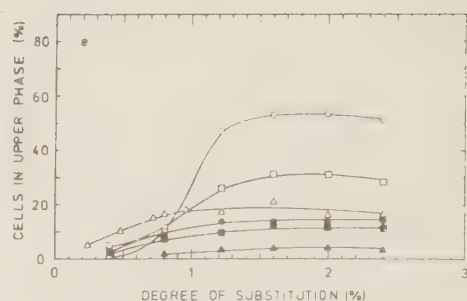
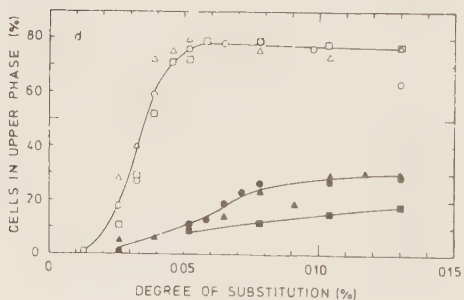
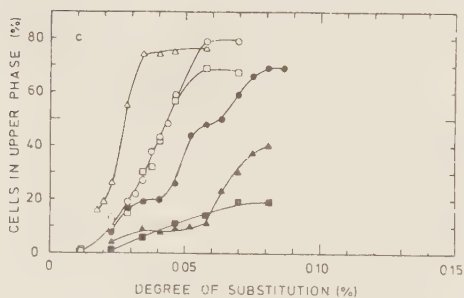
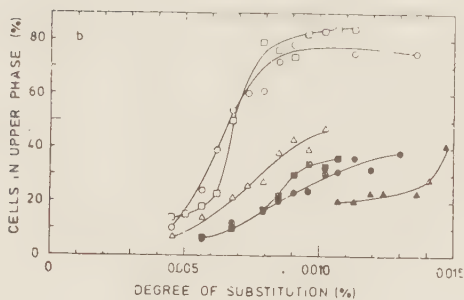
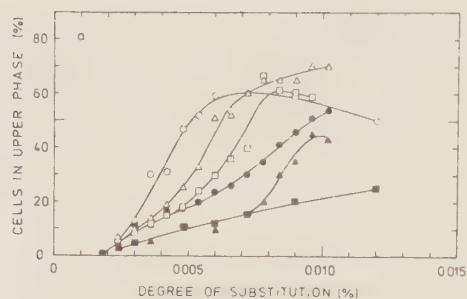


FIG. 2. The percentage of erythrocytes in upper phase as function of the percentage esterified PEG end groups. The erythrocytes came from:  $\circ$ , dog;  $\Delta$ , rat;  $\square$ , guinea pig;  $\bullet$ , sheep;  $\blacksquare$ , rabbit;  $\blacktriangle$ , human. PEG was esterified with (a) palmitic acid; (b) oleic acid; (c) linoleic acid; (d) linolenic acid; and (e) deoxycholic acid. Please notice the different scales on the abscissa.

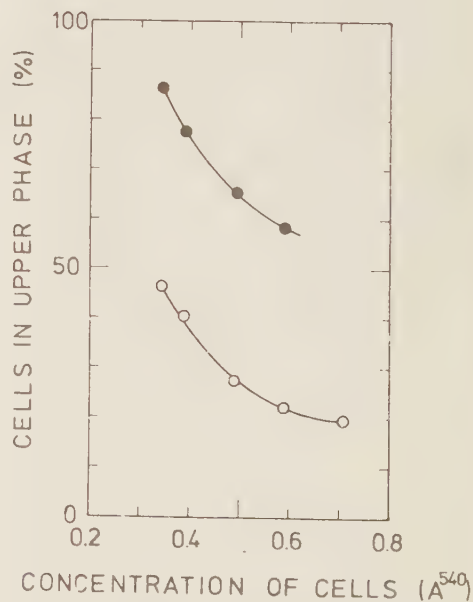


FIG. 3. The dependence of partition on the concentration of erythrocytes from sheep.  $\circ$ , 0.006% of the PEG end groups esterified with palmitic acid; resp.  $\bullet$ , 0.012%.

lower, dextran-rich phase. When the cells are PEG-covered the surface resembles the chemical nature of the PEG-rich, upper phase and the cells will have a stronger affinity for this phase. The results show that this binding is strongly dependent on the chemical structure of the fatty acid ester of PEG. The saturated acid has the highest affinity for the cells. The introduction of double-bonds lower this affinity, especially when the acid contains more than one double-bond. The reason is probably that the reduced flexibility of the unsaturated acid due to the hindered rotation around the double-bond reduces the possibilities of contact. The two hydroxyl groups in deoxycholate are probably responsible for the low affinity of PEG deoxycholate for erythrocytes.

The ability of PEG-esters to bind to erythrocytes is species-dependent. This indicates that cells of different origin may differ in the number of binding sites or in binding strength. From the present results it is not possible to conclude whether the cells from different species have the same number of binding sites or not. The large variations in the slopes of the extraction curves indicates, however, that the binding strength varies between the species.

Erythrocytes from dog, rat and guinea pig fall under one group of cells with high affinity for PEG-ester. These have almost the same amount of phosphatidyl choline (46.9%, 47.5% resp. 41.1% of total phospholipids) and sphingomyelin (10.8%, 12.8% resp. 11.1%) in their membranes. The group of erythrocytes with low affinity for PEG-esters has a higher relative amount of sphingomyelin (rabbit 19.0%, sheep 51.0% and human 26.9%) and a lower relative amount of phosphatidyl choline (rabbit 33.9%, sheep 0.0% and human 28.9%)<sup>8</sup>. It is therefore plausible that these two phospholipids are constituents of the binding sites. This interpretation is supported by the recent studies which indicate that phosphatidyl choline and sphingomyelin are constituents of the outer half of the bilayer leaflet<sup>9</sup>.

Since the PEG-ester has no electrical charge the binding must be purely hydrophobic. The main interaction must be with the hydrocarbon chains of the lipids which are arranged perpendicular to the cell surface. The fatty acid group of the PEG-ester must consequently dip into the outer half of the membrane bilayer and in

that way anchor the PEG-chain on the cell surface.

At certain concentrations of PEG palmitate the erythrocytes aggregated, but this was not the case with the other PEG-esters. The difference between PEG palmitate and the other PEG-esters was that in PEG palmitate 60% of the terminal hydroxyl groups of PEG were esterified while 11% were esterified in PEG oleate, 6% in PEG linolate, 6.5% in PEG linolenate and 8% in PEG deoxycholate. Since PEG palmitate has such a high degree of substitution a considerable number of the linear PEG-chains must have palmitoyl groups at both ends. Two erythrocytes can then share a number of such double-substituted PEG-molecules and in this way aggregate. The dissociation at higher concentrations of PEG palmitate can be explained by the following model: when the number of ester molecules exceeds the number of binding sites on the cell surface the excess is bound to the palmitoyl groups (of the double-substituted PEG-ester) which are sticking out from the cell. Through this binding the main part of these groups will be covered. Since the probability that the covering PEG palmitate mostly is monosubstituted increases, the number of exposed palmitoyl groups are greatly reduced and the reason for the aggregation ceases. In line with this model, totally esterified PEG gave rise to aggregation at all concentrations above 0.0001%.

Partition of cells in phase-systems containing different PEG-bound ligands offers an unique possibility to study the properties of the cell surface. It might be useful in determining binding constants of various ligands to the membrane. This work shows that the chemical composition of the cell membrane is reflected in binding ability towards hydrophobic groups.

## Acknowledgements

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## THE EFFECT OF THE LIPID COMPOSITION ON THE PARTITION OF LIPOSOMES IN AQUEOUS TWO-PHASE SYSTEMS

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### Summary

Liposomes have been partitioned in aqueous two-phase systems consisting of water, dextran, poly(ethylene glycol), salt and buffer. Liposomes were used as a model system in order to determine the contribution of the lipids on the partition of membrane particles. The liposomes were composed of phospholipids with different polar head groups and different degrees of unsaturation. The role of cholesterol was also investigated.

The polar head group of the phospholipid plays a dominant role in determining the partition of liposomes, while the degree of unsaturation is of less importance, thereby indicating that partition in two-phase systems is a surface dependent method. Incorporation of cholesterol in liposomes reduces differences in partition between liposomes of various composition.

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### Introduction

Aqueous two-phase systems [1] have been widely used in the separation of membrane particles [2]. This method utilizes differences in surface properties for separation, rather than differences in size and density. These surface properties include charge and hydrophobicity.

A difference in partition has earlier been found for red blood cells from various species [3], where cells with a higher amount of phosphatidylcholine compared to sphingomyelin have a different partition than have cells with a lower amount. A correlation between the ratio poly/mono-unsaturated fatty acids and the partition of red blood cells from different species has also been reported [4]. Membrane vesicles from spinach chloroplasts with different chlorophyll *a/b* ratios have also been separated by phase partition [5].

Since the cell membrane contains a large amount of lipids, it is of interest to see the effect of each lipid on the partition behaviour and thereby determine



the contribution of the lipids on the partition of membrane particles. For this purpose liposomes were used as a model system. Liposomes were made with different polar head groups and with different degrees of unsaturation of the phospholipids. Cholesterol was also incorporated in some of the liposomes.

This investigation shows that the polar head group of the lipid is a dominant factor in determining the partition, while the degree of unsaturation and the cholesterol content are of less importance.

## Materials and Methods

Dextran 40, batch No 4743,  $M_r = 42\,000$  and dextran 500, batch No 5556,  $M_r = 500\,000$  were supplied by Pharmacia, Uppsala, Sweden. Poly(ethylene glycol),  $M_r = 6000$  was obtained from Union Carbide, New York as Carbowax. The poly(ethylene glycol) ester was synthesized by Dr. Göte Johansson [3]. L- $\alpha$ -Phosphatidylinositol (soya bean), purity 98%, DL- $\alpha$ -phosphatidylcholine-dipalmitoyl, 99% purity, L- $\alpha$ -phosphatidylserine (bovine brain), 98% purity, and sphingomyelin (bovine brain) were obtained from Sigma. Egg phosphatidylcholine and soya bean phosphatidylcholine were generous gifts from Dr. Björn Åkesson [6]. Cholesten-(5)-ol-(3 $\beta$ ), purity 99% was obtained from Merck. [ $^{14}\text{C}$ ]phosphatidylcholine, 50 mCi/mmol and [ $^{14}\text{C}$ ]cholesterol 54 mCi/mmol, purity of both 99%, were from New England Nuclear.

Preparation of liposomes was done according to Huang and Thompson [7]. 14  $\mu\text{mol}$  of lipid were dissolved in chloroform/methanol mixtures in a 5 ml flask and 4 nmol [ $^{14}\text{C}$ ]phosphatidylcholine or 4 nmol [ $^{14}\text{C}$ ]cholesterol were added. The solvent was evaporated under a stream of  $\text{N}_2$ . The vessel was placed in a desiccator under reduced pressure overnight. 1 ml of 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH = 7, was added and the mixture was sonicated under  $\text{N}_2$  above the transition temperature in a Bransonic Cleaning Bath 12 until clearness of the solution. The solution was fractionated on a column of Sepharose 4B, 20  $\times$  2 cm, at 4°C, separating multilayered from the bilayer liposomes. The turbidity and the radioactivity of the eluate were measured. The same results were obtained with radioactively labelled phosphatidylcholine as with labelled cholesterol. The turbidity was measured by a ultra-violet monitor and the radioactivity by a liquid scintillation counter with toluene/Triton X-100/omnifluor, 500 ml : 250 ml : 4.25 g, as a scintillator. The large vesicles which are surrounded by many bilayers are referred to multilayered liposomes in the text, while the small liposomes which are surrounded by only one bilayer are called bilayer liposomes.

Preparation of phase systems: The phase systems used contained 4% (w/w) poly(ethylene glycol) and 8% (w/w) dextran 40 or 5% (w/w) dextran 500. Phase systems of total weight 5 g were prepared by mixing 2 g of a 20% (w/w) dextran 40 solution or 1.25 g of a 20% (w/w) dextran 500 solution with 0.5 g of a 40% (w/w) poly(ethylene glycol) solution and then adding the appropriate salt and buffer to the final concentrations. One ml of liposome suspension was included in these 5 g. The phases were mixed by 30 inversions and then allowed to separate for 25 min (with dextran 40) or 30 min (with dextran 500). All partitions were done at room temperature, 22°C. After separation, 1 ml of the upper, poly(ethylene glycol)-rich, phase and 1 ml of the lower, dextran-rich, phase were withdrawn and the radioactivity of these was measured.



## Results and Discussion

Liposomes of different composition have been partitioned in phase systems composed of 5% (w/w) dextran 500 and 4% (w/w) poly(ethylene glycol) 6000 with (a) 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer, pH 7, or (b) 0.11 mol/kg sodium phosphate buffer, pH 7, and 0.03 mol/kg NaCl. Phase system a has a negative potential [8], i.e., the upper phase is more negative than the lower phase. Phase system b has a positive potential, i.e. the upper phase is more positive than the lower phase. The distribution of the different liposomes between the two phases can be seen in Table I. In this Table one can compare the partition of liposomes having different polar head groups and different degrees of unsaturation of the phospholipids in phase systems with opposite potential.

### *Effect of polar head group*

Both multilayered and bilayer liposomes composed of phosphatidylcholine or sphingomyelin have a slightly but significantly higher partition in phase system a than in phase system b, i.e., they behave as if they carried a positive surface charge (Table I). Both multilayered and bilayer liposomes of phosphatidylserine or phosphatidylinositol have a lower partition in phase system a than in phase system b. This shows that the phase systems can detect a negative surface charge for both phosphatidylserine and phosphatidylinositol liposomes.

Incorporation of cholesterol in molar ratio 1 : 1 in liposomes of phosphatidylcholine or sphingomyelin gives larger differences in partition between phase systems of opposite potential, i.e., the positive surface charge seems to increase. The charge of the liposomes which is registered by the phase systems is thus not the same charge as the sum of the positive and the negative charges from each lipid.

The surface charge can thus be measured qualitatively by partitioning in phase systems with positive and negative potentials and comparing the results. The partition of charged liposomes can be directed by salts, as has been shown earlier for proteins and cells [1].

Phosphatidylinositol liposomes have a higher partition than phosphatidylserine liposomes (Table I). The polar head group therefore seems to be important for the partition behaviour even for liposomes with a negative surface charge.

Partition of liposomes composed of mixtures of phosphatidylcholine and phosphatidylserine shows two effects. 1, both multilayered and bilayer liposomes of phosphatidylcholine : phosphatidylserine in molar ratios 4 : 1, 1 : 1 and 1 : 4 have negative surface charge, since they have a lower partition in phase system a than in phase system b. 2, In the phase system with negative potential (phase system a) the higher the molar ratio of phosphatidylserine, the more the liposomes favour the lower phase. In contrast, in those phase systems with positive potential, liposomes with phosphatidylcholine : phosphatidylserine in molar ratio 4 : 1 favour the upper phase more than liposomes composed of only one of the phospholipids.

There thus seem to be two tendencies working in opposite direction, one depending on the surface charge and one depending on the structure of the

TABLE I

The distribution of liposomes between the two phases in 5% (w/w) dextran 500, 4% (w/w) poly(ethylene glycol) and (a) 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer or (b) 0.03 mol/kg NaCl and 0.11 mol/kg sodium phosphate buffer. These phase systems have opposite potentials. Each 5-g phase system contain 0.19  $\mu$ mol phospholipid. Values in parentheses are for bilayer liposomes and values without parentheses are for multilayer liposomes.

| Liposome composition                                                   | Percentage of liposomes |                  |                    |
|------------------------------------------------------------------------|-------------------------|------------------|--------------------|
|                                                                        | in the upper phase      | at the interface | in the lower phase |
| Soya bean phosphatidylcholine                                          |                         |                  |                    |
| a                                                                      | 8 (51)                  | 76 (24)          | 16 (25)            |
| b                                                                      | 2 (41)                  | 64 (23)          | 34 (36)            |
| Soya bean phosphatidylcholine/cholesterol; molar ratio 1 : 1           |                         |                  |                    |
| a                                                                      | (4)                     | (80)             | (16)               |
| b                                                                      | (1)                     | (45)             | (54)               |
| Egg phosphatidylcholine                                                |                         |                  |                    |
| a                                                                      | 5 (29)                  | 74 (41)          | 21 (30)            |
| b                                                                      | 3 (25)                  | 81 (42)          | 16 (33)            |
| Dipalmitoylphosphatidylcholine                                         |                         |                  |                    |
| a                                                                      | 13 (5)                  | 64 (61)          | 23 (34)            |
| b                                                                      | 2 (4)                   | 64 (55)          | 34 (41)            |
| Dipalmitoylphosphatidylcholine/cholesterol; molar ratio 1 : 1          |                         |                  |                    |
| a                                                                      | 8 (5)                   | 79 (77)          | 13 (18)            |
| b                                                                      | 0 (1)                   | 52 (53)          | 48 (46)            |
| Sphingomyelin                                                          |                         |                  |                    |
| a                                                                      | 1 (4)                   | 63 (61)          | 36 (35)            |
| b                                                                      | 1 (2)                   | 65 (58)          | 34 (40)            |
| Sphingomyelin/cholesterol; molar ratio 1 : 1                           |                         |                  |                    |
| a                                                                      | 11 (7)                  | 74 (70)          | 15 (23)            |
| b                                                                      | 1 (1)                   | 62 (48)          | 37 (51)            |
| Dipalmitoylphosphatidylcholine/phosphatidylserine; molar ratios:       |                         |                  |                    |
| 4 : 1                                                                  |                         |                  |                    |
| a                                                                      | 1 (28)                  | 41 (11)          | 58 (61)            |
| b                                                                      | 4 (58)                  | 77 (26)          | 19 (16)            |
| 1 : 1                                                                  |                         |                  |                    |
| a                                                                      | 1 (5)                   | 8 (11)           | 91 (84)            |
| b                                                                      | 2 (43)                  | 58 (27)          | 40 (30)            |
| 1 : 4                                                                  |                         |                  |                    |
| a                                                                      | 1 (5)                   | 0 (5)            | 99 (90)            |
| b                                                                      | 2 (20)                  | 46 (31)          | 52 (49)            |
| Phosphatidylserine                                                     |                         |                  |                    |
| a                                                                      | 1 (2)                   | 0 (2)            | 99 (96)            |
| b                                                                      | 1 (2)                   | 28 (25)          | 71 (73)            |
| Dipalmitoylphosphatidylcholine/phosphatidylinositol; molar ratio 1 : 1 |                         |                  |                    |
| a                                                                      | 1                       | 7                | 92                 |
| b                                                                      | 3                       | 64               | 33                 |
| Phosphatidylinositol                                                   |                         |                  |                    |
| a                                                                      | (20)                    | (8)              | (72)               |
| b                                                                      | (47)                    | (6)              | (47)               |

polar head group. This might cause difficulties in predicting the partition of mixed phospholipid bilayers.

#### *Effect of degree of unsaturation*

In Table I we can study also the effect of the degree of unsaturation of the phospholipids. Bilayer phosphatidylcholine liposomes with the most unsaturated lipids have a higher partition than bilayer liposomes with saturated lipids. Soya bean phosphatidylcholine has 21% saturated, 23% monounsaturated and 56%

polyunsaturated fatty acyl chains [9]. Egg phosphatidylcholine has 48% saturated, 31% monounsaturated and 20% polyunsaturated fatty acyl chains [10]. Dipalmitoylphosphatidylcholine has 100% saturated fatty acyl chains. This difference in partition between saturated and unsaturated phosphatidylcholines cannot be found for multilayered liposomes. Incorporation of cholesterol in bilayer liposomes of soya bean phosphatidylcholine or dipalmitoylphosphatidylcholine removes the differences in partition between saturated and unsaturated liposomes (Table I). Incorporation of cholesterol in liposomes of phosphatidylcholine and sphingomyelin also removes the differences in partition between these two types of liposomes.

The fact that the degree of unsaturation is reflected in partition of bilayer vesicles but not in partition of multilayered liposomes might be explained by the higher radius of curvature for bilayer liposomes compared to multilayered [11]. The hydrophobic part of the membrane will be more exposed to the surroundings in the case of bilayer vesicles. Cholesterol is supposed to reduce the membrane fluidity and this may be the explanation to the equalizing effect of cholesterol on the partition [12].

### *Effect of poly(ethylene glycol) palmitate*

Poly(ethylene glycol) palmitate has earlier been used in experiments with erythrocytes [3]. In phase systems with 8% (w/w) dextran 40, 4% (w/w) poly(ethylene glycol) 6000, 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer, pH 7, the liposomes are at the interface and/or in the lower, dextran-rich phase. If some of the terminal hydroxylgroups of poly(ethylene glycol) are esterified with palmitic acid the liposomes will be found in the upper phase.

Neither for multilayered nor for bilayer phosphatidylcholine liposomes were there any differences in partition when the degree of unsaturation of the phospholipid was changed. Fig. 1. Incorporation of cholesterol in phosphatidylcholine liposomes in molar ratio 1 : 1 has no effect on the partition of multilayered liposomes. Bilayer vesicles with cholesterol in molar ratio 1 : 1 require somewhat less concentration of poly(ethylene glycol) palmitate to get into the upper phase than bilayer liposomes without cholesterol.

For bilayer vesicles the amount of poly(ethylene glycol) palmitate which is required to get 50% of the liposomes into the upper phase is in the order of five poly(ethylene glycol) palmitate molecules per liposome. The corresponding number for erythrocytes was  $5 \cdot 10^6$  molecules per red blood cell. The amount of poly(ethylene glycol) palmitate per mole lipid that is required to get the particles into the upper phase is 10-times higher for erythrocytes than for bilayer liposomes.

Liposomes of different composition i.e. phosphatidylcholine, sphingomyelin, phosphatidylserine and phosphatidylinositol are compared in Figs. 1, 2 and 3. Multilayered phosphatidylcholine liposomes require the lowest poly(ethylene glycol) palmitate concentration to get into the upper phase. Fig. 1a. At somewhat higher concentration of poly(ethylene glycol) palmitate, bilayer vesicles will be found in the upper phase. Fig. 1b. Liposomes of sphingomyelin require higher concentrations of poly(ethylene glycol) palmitate to get into the upper phase than phosphatidylcholine liposomes (Fig. 2). The liposomes in Fig. 2

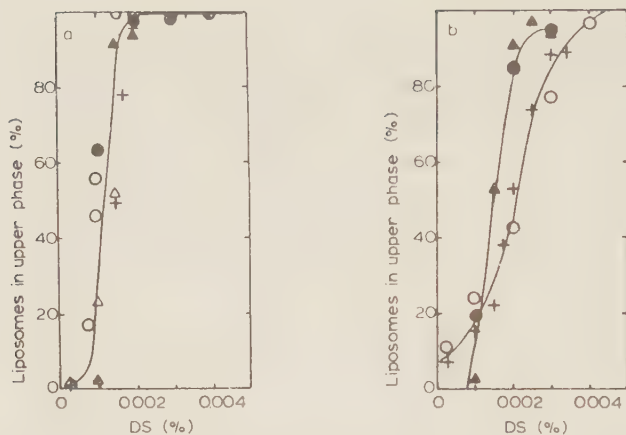


Fig. 1. Saturated and unsaturated phosphatidylcholine liposomes in phase systems containing poly(ethylene glycol) palmitate. The percentage of phosphatidylcholine liposomes in the upper phase as function of the percentage poly(ethylene glycol) end groups esterified with palmitic acid, DS, degree of substitution. The phase systems were composed of 8% (w/w) dextran 40, 4% (w/w) poly(ethylene glycol) 6000, 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer. Each 5 g phase system contains 0.35  $\mu$ mol phospholipid. ○, soya bean phosphatidylcholine; +, egg phosphatidylcholine; △, dipalmitoylphosphatidylcholine, ▲, dipalmitoylphosphatidylcholine/cholesterol, molar ratio 1 : 1 and ●, soya bean phosphatidylcholine/cholesterol, molar ratio 1 : 1. Fig. 1a shows multilayered liposomes and Fig. 1b bilayer liposomes.

were sonicated until clearness of the solutions, but not separated according to size on a column, in order to be sure that no changes for the partition experiments occurred during the separation. The sphingomyelin in Fig. 2 was from bovine brain (66% saturated, 32% monounsaturated and no polyunsaturated

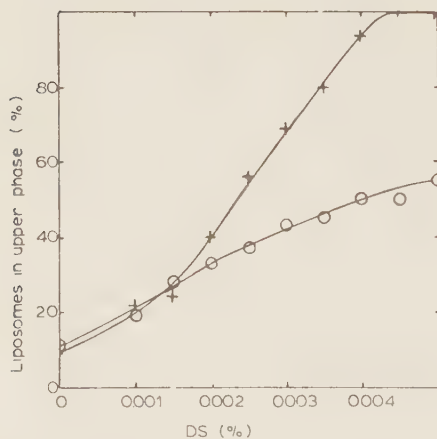


Fig. 2. Sphingomyelin and phosphatidylcholine liposomes in phase systems containing poly(ethylene glycol) palmitate. The percentage of liposomes in the upper phase as function of the percentage poly(ethylene glycol) end groups esterified with palmitic acid, DS. The phase systems were composed of 8% (w/w) dextran 40, 4% (w/w) poly(ethylene glycol) 6000, 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer. Each 5 g phase system contains 0.35  $\mu$ mol phospholipid. The liposomes were not separated according to size and were thus a mixture of multilayered and bilayer liposomes. ○, sphingomyelin and +, dipalmitoylphosphatidylcholine.



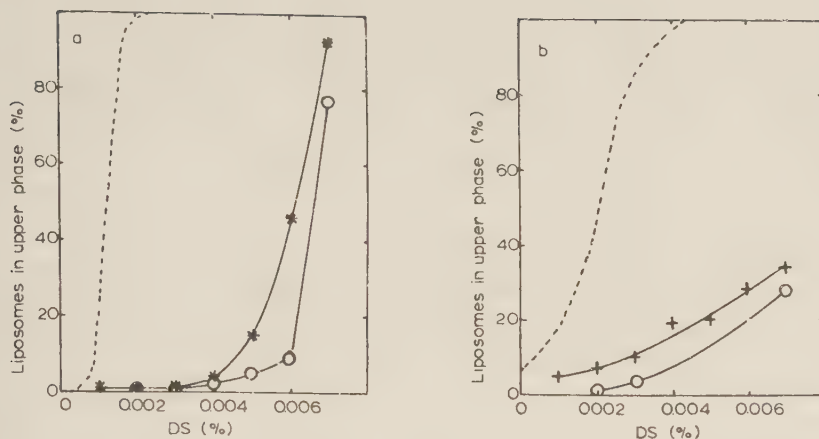


Fig. 3. Negatively-charged liposomes in phase systems containing poly(ethylene glycol) palmitate. The percentage of liposomes in the upper phase as function of the percentage poly(ethylene glycol) end groups esterified with palmitic acid, D.S. The phase systems contain 8% (w/w) dextran 40, 4% (w/w) poly(ethylene glycol) 6000, 0.15 mol/kg NaCl and 0.01 mol/kg sodium phosphate buffer. Each 5 g phase system contains 0.35  $\mu$ mol phospholipid. \*, phosphatidylinositol : phosphatidylcholine, molar ratio 1 : 1; +, phosphatidylinositol and  $\circ$ , phosphatidylserine. The dotted line represents the corresponding curve for phosphatidylcholine liposomes from Fig. 1. Fig. 3a shows multilayered liposomes and Fig. 3b shows bilayer liposomes.

fatty acyl chains [13]) and the phosphatidylcholine was dipalmitoylphosphatidylcholine. Incorporation of cholesterol in sphingomyelin liposomes in molar ratio 1 : 1 removes this difference in partition between phosphatidylcholine and sphingomyelin liposomes. The partition behaviour for sphingomyelin/cholesterol liposomes becomes similar to that of phosphatidylcholine liposomes (not shown in figures). At even higher concentrations of poly(ethylene glycol) palmitate the negatively charged liposomes will be found in the upper phase. Fig. 3.

Differences in liposome composition can therefore be detected in phase systems containing poly(ethylene glycol) palmitate. The polar head group of the phospholipid is also here the dominant factor in determining the partition of liposomes. The degree of unsaturation of the phospholipid has negligible influence on the partition in this case. Differences in partition between liposomes of phosphoglycerides and sphingolipids with the same polar head group can be found with poly(ethylene glycol) palmitate.

It is reasonable to assume that the fatty acyl group of poly(ethylene glycol) palmitate dips down into the bilayer and in that way anchors the poly(ethylene glycol) chain. The results of this investigation shows that the strength of this binding is more dependent of the polar head group than of the fatty acyl part of the phospholipid.

## Conclusions

### *Dextran-poly(ethylene glycol) phase systems*

1. The polar head group of the phospholipid is the dominant factor in determining the partition of liposomes.

2. The charge of the liposomes can be detected. The surface charge of the liposomes is not the same charge as the sum of the charges of the phospholipids.
3. The degrees of unsaturation of the phospholipids are of less importance for the partition behaviour.
4. Cholesterol plays a minor role in determining the partition.

#### *Dextran-poly(ethylene glycol)-palmitate phase system*

1. Poly(ethylene glycol) palmitate has affinity for lipid bilayers and can change the partition of liposomes from the lower phase to the upper phase.
2. Poly(ethylene glycol) palmitate can detect differences in surface properties of liposomes. The polar head group of the phospholipid is the dominant factor in determining the partition.

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HYDROPHOBIC AFFINITY PARTITION OF LIPOSOMES IN  
AQUEOUS TWO-PHASE SYSTEMS.

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## INTRODUCTION.

Aqueous two-phase systems consisting of the two polymers dextran and poly(ethylene glycol) are a useful tool in the separation and study of cells and membrane particles [1,2] . Both phases have a high content of water, 80-95%, and are therefore very mild and gentle for biological materials. Salts and sugars may be added to suitable concentrations.

Hydrophobic affinity partition in aqueous two-phase systems with fatty acids covalently attached to poly(ethylene glycol) has been used for selective partition of both proteins [3,4,5] and membranes [6,7,8]. Material with affinity for the poly(ethylene glycol)-bound fatty acids will change their partition from the lower dextran-rich phase to the upper poly(ethylene glycol)-rich phase.

Liposomes have been partitioned in phase systems with poly(ethylene glycol) esterified with palmitic acid [9]. Differences in partition behaviour were found when the polar head groups of the phospholipids were varied but not when the degree of unsaturation was varied.

It has been shown with chloroplast membranes that the length of the poly(ethylene glycol)-bound fatty acid is of importance [6]. Intact and broken chloroplasts were separated when capric acid was attached to poly(ethylene glycol), but not when longer fatty acids were used.

This investigation was done in order to see whether the effect of poly(ethylene glycol)-bound fatty acids of varying length was reflected in partition of liposomes of different composition. Therefore, dilauroyl phosphatidylcholine, dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine and egg phosphatidylcholine as well as phosphatidylserine liposo-

mes were partitioned in phase systems with poly(ethylene glycol) esterified with capric acid, lauric acid, myristic acid, palmitic acid and stearic acid.

The longer the poly(ethylene glycol)-bound fatty acid, the less amount of it is required for extraction of liposomes from the lower phase.

The results show that neither differences in length nor degree of unsaturation of the liposomal phospholipids give partition differences with a poly(ethylene glycol)-bound fatty acid of varying length. In contrast, variations in the polar part of the liposomes dominate the partition. Differences in partition of phosphatidylcholine and phosphatidylserine liposomes were more pronounced when the poly(ethylene glycol)-bound fatty acid was decreased in length from stearic acid to capric acid.

## EXPERIMENTAL.

Dextran 40, batch No 5970,  $M_r=42000$  was supplied by Pharmacia, Uppsala, Sweden. Poly(ethylene glycol),  $M_r=6000$  was obtained from Union Carbide, New York as Carbowax. The poly(ethylene glycol)-esters were synthesized by Dr Göte Johansson [4]. L- $\alpha$ -phosphatidylcholine dilauroyl, purity 98%, L- $\alpha$ -phosphatidylcholine dimyristoyl, purity 98%, DL- $\alpha$ -phosphatidylcholine dipalmitoyl, purity 99%, L- $\alpha$ -phosphatidylcholine distearoyl, purity 99% and L- $\alpha$ -phosphatidylserine (bovine brain), purity 98-99% were obtained from Sigma. Egg phosphatidylcholine were prepared according to [10]. ( $^{14}\text{C}$ )-phosphatidylcholine 50 mCi/mmol, purity 99% was from New England Nuclear.

Preparation of liposomes was done according to Huang and Thompson [11]: 14  $\mu\text{mol}$  of lipid were dissolved in chloroform in a 5 ml flask and 4 nmol ( $^{14}\text{C}$ )-phosphatidylcholine was added. The solvent was evaporated under a stream of  $\text{N}_2$ . The vessel was placed in a desiccator under reduced pressure. One ml of 0.15 M NaCl and 0.01 M Na-phosphate buffer, pH=7, was added and the mixture was sonicated under  $\text{N}_2$  above the transition temperature in a Bransonic Cleaning Bath 12 for 15 minutes. The resulting solution contained mostly multilayered liposomes.

Preparation of phase systems: The 5 g phase system used contained 8% (w/w) dextran 40, 4% (w/w) poly(ethylene glycol) 6000, 0.15 M NaCl, 0.01 M Na-phosphate buffer, pH=7, and 0.35  $\mu\text{mol}$  phospholipid. The phases were mixed by 30 inversions and allowed to separate for 25 minutes. All partitions were done at  $22^\circ\text{C}$ . After separation, one ml of the upper, poly(ethylene glycol)-rich phase and one ml of the lower dextran-rich phase were withdrawn and the radioactivity was measured. The amount at the interface was calculated from the total amount in the phase system.

## RESULTS.

In phase systems composed of 8%(w/w) dextran 40, 4%(w/w) poly(ethylene glycol) 6000, 0.15 M NaCl and 0.01 M Na-phosphate buffer, pH=7, the liposomes are to 93-98% in the lower dextran-rich phase. If some of the terminal hydroxyl groups of poly(ethylene glycol) are esterified with fatty acids the liposomes will be found at the interface and in the upper phase.

In fig 1 is shown how liposomes increase their affinity for the interface and the upper phase as the concentration of poly(ethylene glycol)-bound fatty acid increase. The longer the fatty acids of poly(ethylene glycol), the more effective will the extraction of liposomes be. I.e. the lower concentration of poly(ethylene glycol)-bound fatty acid is required for transferring the liposomes from the lower phase. Both for phosphatidylcholine and phosphatidylserine liposomes the effectiveness is in the following order : poly(ethylene glycol) caprate < poly(ethylene glycol) laurate < poly(ethylene glycol) myristate < poly(ethylene glycol) palmitate < poly(ethylene glycol) stearate.

There were no differences in partition behaviour between phosphatidylcholine liposomes with different hydrophobic parts of the bilayer, fig 1. I.e. dilauroyl phosphatidylcholine, dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine and egg phosphatidylcholine liposomes were all extracted from the lower phase at the same concentrations of poly(ethylene glycol)-bound fatty acids.

Table 1 shows the amount of poly(ethylene glycol)-bound fatty acid that is required to get 50% of the liposomes at

the interface and in the upper phase. It can be seen that 5-15 times more poly(ethylene glycol)-bound fatty acid is required to extract phosphatidylserine liposomes compared to phosphatidylcholine liposomes. The ratio increases when shorter fatty acids are used. I.e. the shorter the poly(ethylene glycol)-bound fatty acid, the higher concentration will be required to extract 50% of phosphatidylserine liposomes compared to phosphatidylcholine liposomes from the lower phase.

In fig 2  $-\log DS$  for extraction of 50% of the liposomes from the lower phase is plotted against the number of carbon atoms of the fatty acid that is attached to poly(ethylene glycol).



## DISCUSSION.

The effect of poly(ethylene glycol)-bound fatty acids on the partition of liposomes is explained by assuming an interaction of the poly(ethylene glycol)-bound fatty acid with the liposomal bilayer. Owing to a weaker interaction of the shorter fatty acids, these are required in higher concentrations for extraction of liposomes from the lower phase. The deeper the poly(ethylene glycol)-bound fatty acids dip down into the liposomal bilayer, the stronger are the hydrophobic interactions between longer fatty acids and the hydrophobic part of the bilayer.

This interaction is independent of the nature of the hydrophobic part of the liposomes, since there were no differences in partition of liposomes neither with different length nor with different degree of unsaturation of the phospholipid fatty acids. The phospholipids were chosen to have transition temperatures below (egg phosphatidylcholine and dilauroyl phosphatidylcholine), almost the same (dimyristoyl phosphatidylcholine) and above (dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine) the temperature during the partition. Thus the fluidity of the bilayer does not influence the partition behaviour.

The polar head group of the phospholipid plays a dominant role in determining the partition of liposomes when poly(ethylene glycol)-bound fatty acids are present. Since there were no partition differences when the hydrophobic part was varied it is instead the accessibility to the hydrophobic part that is important for the interaction. Both electrostatic and steric factors may contribute to this effect. In the case of biological membranes, proteins might also influence the partition.

A simple way to look upon this interaction is to regard the binding of a poly(ethylene glycol)-bound fatty acid F to a binding site B on the liposome which results in a complex BF. Thus  $B + F \rightleftharpoons BF$ . The equilibrium equation is :

$K = \frac{[BF]}{[B] \cdot [F]}$ . In the simplest case with only one poly(ethylene glycol)-bound fatty acid on each liposome that is removed from the lower phase and when 50% of the liposomes are extracted from the lower phase  $K = \frac{1}{[F]}$ . In this case  $[F]$  is directly proportional to DS. Thus  $K \sim \frac{1}{DS}$ .

The binding of a poly(ethylene glycol)-bound fatty acid to the hydrophobic part of the bilayer may also be regarded as partition of a hydrocarbon between an aqueous phase and an organic phase.  $\Delta G$  for such a process is proportional to the length,  $n$ , of the hydrocarbon i.e. in this case the length of the poly(ethylene glycol)-bound fatty acid [12]. Thus  $\Delta G = -RT \ln K \sim \text{constant} \cdot n$ . And with 50% of the liposomes at the interface and in the upper phase:  $-RT \ln \frac{1}{DS} \sim \text{constant} \cdot n$  which means that  $\log DS \sim \text{constant} \cdot n$ . This is also seen in fig 2 where there is an almost linear relation between  $-\log DS$  and the number of carbon atoms of the poly(ethylene glycol)-bound fatty acid until it reaches the critical number of 16 where a further increase has very little effect.

Although shorter poly(ethylene glycol)-bound fatty acids have to be used in higher concentrations they are still very useful for separation purposes. This is shown in the facilitated extraction of phosphatidylcholine liposomes compared to phosphatidylserine liposomes from the lower phase as the poly(ethylene glycol)-bound fatty acid decreases in length (as the ratio in table 1 shows). It was also noticed with chloroplast membranes [6] where intact and broken chloroplasts were separated when capric acid was attached to poly(ethylene glycol) but not when longer fatty acids were used. Thus one ought to try several lengths of poly(ethylene glycol)-bound fatty acids in order to achieve the best separation.

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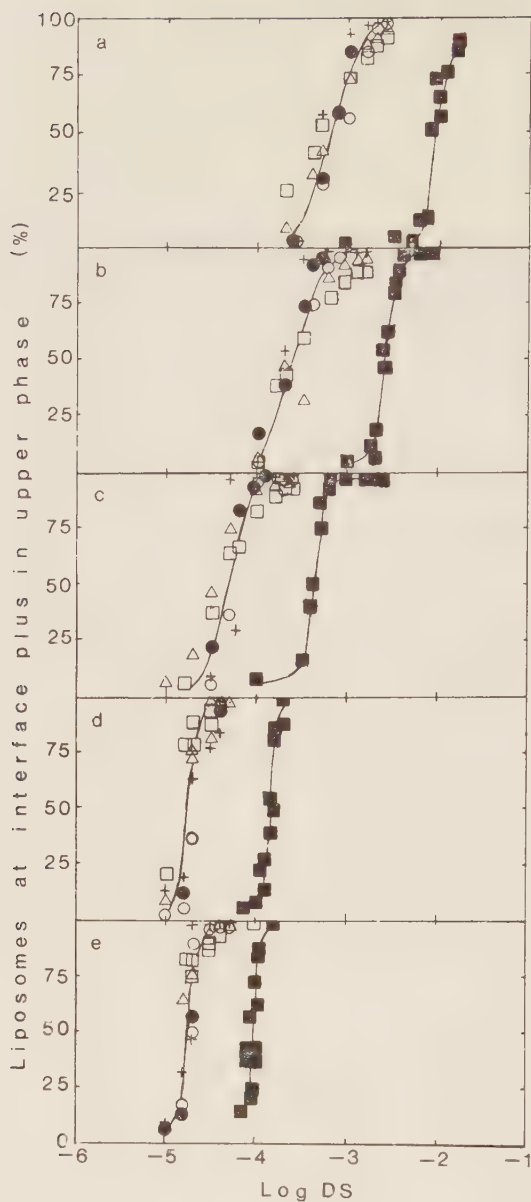


Fig 1.

The percentage of liposomes at interface plus in upper phase as function of the fraction of poly(ethylene glycol) end groups esterified with fatty acids, DS, degree of substitution. +, dilauroyl phosphatidylcholine; o, dimyristoyl phosphatidylcholine;  $\Delta$ , dipalmitoyl phosphatidylcholine;  $\square$ , distearoyl phosphatidylcholine;  $\bullet$ , egg phosphatidylcholine and  $\blacksquare$ , phosphatidylserine. Poly(ethylene glycol) is esterified with a) capric acid C:10, b) lauric acid C:12, c) myristic acid C:14, d) palmitic acid C:16 and e) stearic acid C:18.

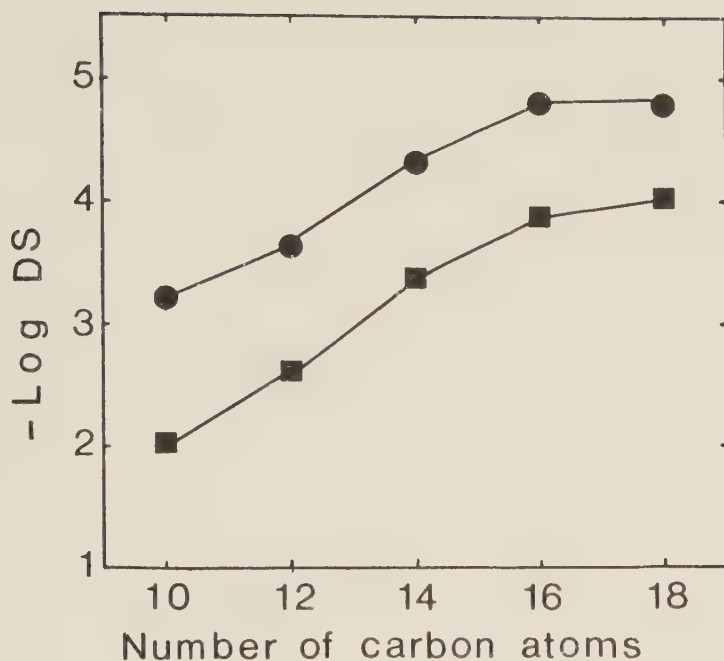


Fig 2.

The fraction of poly(ethylene glycol) end groups esterified with fatty acids, DS, for extraction of 50% of the liposomes from the lower phase as function of the number of carbon atoms of the fatty acids. ●, phosphatidylcholine and ■, phosphatidylserine liposomes.

TABLE 1

The fraction of poly(ethylene glycol) end groups esterified with fatty acids for extraction of 50% of the liposomes from lower phase to interface and upper phase. DS = degree of substitution = 
$$\frac{\text{poly(ethylene glycol)-bound fatty acid}}{\text{poly(ethylene glycol)}}$$

| Fatty acid<br>bound to<br>poly(ethy-<br>lene gly-<br>col) | DS when 50% of the liposomes<br>are at the interface and in<br>the upper phase. |                                        | DS ratio, phos-<br>phatidylserine:<br>phosphatidyl-<br>choline liposo-<br>mes when 50% are<br>at the interface<br>and in the upper<br>phase |
|-----------------------------------------------------------|---------------------------------------------------------------------------------|----------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|
|                                                           | Phosphatidyl-<br>choline lipo-<br>somes                                         | Phosphatidyl-<br>serine lipo-<br>somes |                                                                                                                                             |
| Capric acid C:10                                          | $6.3 \times 10^{-4}$                                                            | $9.6 \times 10^{-3}$                   | 15.2                                                                                                                                        |
| Lauric acid C:12                                          | $2.4 \times 10^{-4}$                                                            | $2.5 \times 10^{-3}$                   | 10.4                                                                                                                                        |
| Myristic acid C:14                                        | $4.7 \times 10^{-5}$                                                            | $4.4 \times 10^{-4}$                   | 9.4                                                                                                                                         |
| Palmitic acid C:16                                        | $1.7 \times 10^{-5}$                                                            | $1.4 \times 10^{-4}$                   | 8.2                                                                                                                                         |
| Stearic acid C:18                                         | $1.7 \times 10^{-5}$                                                            | $9.6 \times 10^{-5}$                   | 5.6                                                                                                                                         |











